

# DNAX Accessory Molecule-1 Mediated Recognition of Freshly Isolated Ovarian Carcinoma by Resting Natural Killer Cells

Mattias Carlsten,<sup>1,2</sup> Niklas K. Björkström,<sup>1</sup> Håkan Norell,<sup>2</sup> Yenan Bryceson,<sup>1</sup> Thorbald van Hall,<sup>1,4</sup> Bettina C. Baumann,<sup>1</sup> Mikael Hanson,<sup>2</sup> Kjell Schedvins,<sup>3</sup> Rolf Kiessling,<sup>2</sup> Hans-Gustaf Ljunggren,<sup>1</sup> and Karl-Johan Malmberg<sup>1</sup>

<sup>1</sup>Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge; <sup>2</sup>Immune and Gene Therapy Laboratory, Cancer Center Karolinska, Department of Oncology and Pathology, Karolinska Institutet; <sup>3</sup>Department of Obstetrics and Gynecology, Karolinska University Hospital Solna, Stockholm, Sweden and <sup>4</sup>Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands

## Abstract

Although natural killer (NK) cells are well known for their ability to kill tumors, few studies have addressed the interactions between resting (nonactivated) NK cells and freshly isolated human tumors. Here, we show that human leukocyte antigen class I<sup>low</sup> tumor cells isolated directly from patients with advanced ovarian carcinoma trigger degranulation by resting allogeneic NK cells. This was paralleled by induction of granzyme B and caspase-6 activities in the tumor cells and significant tumor cell lysis. Ovarian carcinoma cells displayed ubiquitous expression of the DNAX accessory molecule-1 (DNAM-1) ligand PVR and sparse/heterogeneous expression of the NKG2D ligands MICA/MICB and ULBP1, ULBP2, and ULBP3. In line with the NK receptor ligand expression profiles, antibody-mediated blockade of activating receptor pathways revealed a dominant role for DNAM-1 and a complementary contribution of NKG2D signaling in tumor cell recognition. These results show that resting NK cells are capable of directly recognizing freshly isolated human tumor cells and identify ovarian carcinoma as a potential target for adoptive NK cell-based immunotherapy. [Cancer Res 2007;67(3):1317–25]

## Introduction

Natural killer (NK) cells are regulated by a balance between activating and inhibitory receptor signals. Inhibition is largely mediated via interaction of human leukocyte antigen (HLA) class I molecules with killer cell immunoglobulin-like receptors (KIR) and/or CD94/NKG2A (1, 2). NK cell activation is dependent on, and fine tuned by, signaling through a wide array of activating and costimulatory NK cell receptors (NKR), including NKG2D, DNAX accessory molecule-1 (DNAM-1), 2B4, NTB-A, CRACC, CD2, CD59, NKp80, CD94/NKG2C, and the natural cytotoxicity receptors (NCR; NKp30, NKp44, and NKp46; ref. 3). Certain combinations of activating receptors may synergize and trigger NK cell activation more efficiently than others (4). Moreover, different activating receptors may contribute to qualitatively distinct events. For instance, it was recently shown that target cell adhesion, perforin

polarization, and NK cell degranulation are guided by different receptor-ligand interactions (5). Efficient cytolytic responses were the result of a polarized degranulation toward the target and, therefore, required signaling through a specific combination of activating receptors and coreceptors (5).

NK cells have a well-documented capacity to kill a wide variety of tumors. Yet, few studies have explored the interactions between resting (nonactivated) NK cells and freshly isolated human tumors. Such studies have been hampered by technical difficulties in monitoring specific lysis of tumor cells within heterogeneous patient-derived cell populations. Thus, the receptor-ligand interactions that govern the activation of resting human NK cells in response to freshly isolated tumor cells with a complex NKR ligand repertoire remain poorly understood. The capacity of resting NK cells to kill freshly isolated human tumors is important to explore because several protocols of NK cell-based immunotherapy that are currently evaluated in the clinic involve infusion of non-manipulated or short-term interleukin-2 (IL-2)-activated NK cells to patients with hematopoietic and solid tumors (6, 7). Additionally, the repertoire of donor-derived NK cells that mature following allogeneic stem cell transplantation and that has been implicated in the graft-versus-leukemia (GVL) effect against acute myelogenous leukemia (AML; ref. 8) is likely to resemble a resting (nonactivated) phenotype.

To study the interactions between resting NK cells and freshly isolated human tumors, we chose to investigate NK cell recognition of ovarian carcinoma. This tumor type is accessible as single-cell suspensions in ascites fluid of patients with advanced disease. Furthermore, ovarian carcinoma cells often display reduced levels of HLA class I expression, which facilitates studies of NK cell-activating receptor-ligand interactions (9, 10). We show here that resting allogeneic human NK cells recognize and kill freshly isolated ovarian carcinoma cells. Triggering of NK cell cytotoxicity was dependent on signaling through DNAM-1 receptors with a complementary contribution of NKG2D receptors, reflecting the surface expression of corresponding NKR ligands on the ovarian carcinoma cells. Patient-derived fibroblasts displayed higher expression of HLA class I and lower expression of ligands for activating NKRs and were not killed by resting NK cells under similar experimental conditions, indicating a preferential recognition of tumor cells. The susceptibility of ovarian carcinoma cells to resting NK cells suggests that this tumor type may be a potential candidate for NK cell-based adoptive immunotherapy.

## Materials and Methods

**Cells.** This study was approved by the institutional ethics committee (Karolinska Institutet, approval number 03-537). The ovarian carcinoma

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** Karl-Johan Malmberg, Center for Infectious Medicine, F59, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden. Phone: 46-8-58589688; Fax: 46-8-7466280; E-mail: kalle.malmberg@ki.se.

©2007 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-06-2264

cells were obtained with informed consent from patients with epithelial ovarian carcinoma subjected to primary surgery. Ovarian carcinoma cells were enriched from peritoneal effusions by density gradient centrifugation (Ficoll-Hypaque, Amersham Biosciences, Uppsala, Sweden) as described previously (11). Cells were frozen in a solution containing 10% DMSO (Sigma-Aldrich, St. Louis, MO) and 90% heat-inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, NY) and stored in liquid nitrogen. For functional assays, CD45<sup>+</sup> cells were depleted (EasySep kit, Stemcell Technologies, Vancouver, British Columbia, Canada) to obtain pure (>95%) tumor cell populations that were used without further processing or culturing. When indicated, short-term (2 weeks) cocultures of ovarian tumor cells and autologous fibroblasts were obtained by seeding cells from CD45-depleted ascites in complete medium (RPMI 1640 supplemented with 100 µg/mL L-glutamine, 10% heat-inactivated FBS, 100 units/mL penicillin G, and 100 µg/mL streptomycin). For isolation of NK cells, buffy coats from healthy donors were separated by density gradient centrifugation (Ficoll-Hypaque). NK cells were then enriched by magnetic bead negative selection (NK selection kit II, Miltenyi Biotec, Bergisch Gladbach, Germany) of the nonadherent fraction. NK cells used in assays were >95% CD3<sup>-</sup>CD56<sup>+</sup>, and the CD3<sup>+</sup> cell contamination was <1%. NK cells were resuspended in complete medium with or without 1,000 IU/mL IL-2 (Proleukin, Chiron Corp., Emeryville, CA) and incubated overnight (18 h) at 37°C. The human erythroleukemia cell line K562 and the mouse mastocytoma cell line P815 (both from the American Type Culture Collection, Manassas, VA) were maintained in complete medium. The HLA class I-deficient EBV-transformed B-cell line 721.221 (.221) and 721.221 cells transfected with HLA Cw3 (.221.Cw3) were kindly provided by E.O. Long (National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD) and P. Parham (Stanford University, Stanford, CA) and maintained in complete medium.

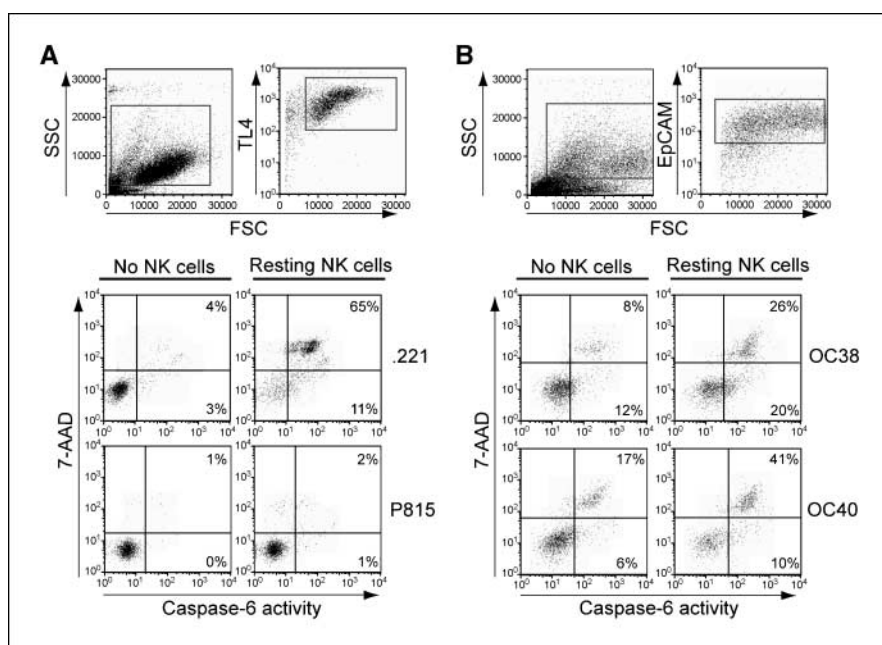
**Antibodies.** The following conjugated monoclonal antibodies (mAb) were used: anti-CD226 (DX11), anti-NKG2D (1D11), anti-CD107a (H4A3), anti-CD14 (MΦP9), anti-CD112 (R2.525), anti-EpCAM (EBA-1), anti-CD90 (5E10), IgG1 (X40), IgG1 (X40), IgG2a (G155-178), and rat anti-mouse IgG1 (X56; Becton Dickinson, Franklin Lakes, NJ); anti-CD3 (UCHT1) and anti-human epithelial antigen (Ber-EP4; DakoCytomation, Glostrup, Denmark); anti-CD56 (N901), anti-NKp30 (Z25), anti-NKp44 (Z231), anti-NKp46 (BAB281), and IgG1 (679.1Mc7; Beckman Coulter, Fullerton, CA); and anti-HLA-ABC (W6/32; Serotec, Oxford, United Kingdom). The following nonconjugated antibodies were used: anti-CD155 (PV404.19; Beckman Coulter); IgG1 (MOPC-21; BioLegend, San Diego, CA); anti-CD96 (NK92.29; Hycult Biotechnology BV, Uden, the Netherlands); and anti-ULBP1 (M295),

anti-ULBP2 (M311), anti-ULBP3 (M551), anti-MICA (M673), and anti-MICB (M362; Amgen, Inc., Seattle, WA). The following nonconjugated antibodies were used for blocking experiments: anti-HLA class I (A6-136, IgM; kindly provided by A. Moretta, University of Genoa, Genoa, Italy), anti-NKG2D (M585; Amgen), anti-CD226 (DX11; Becton Dickinson), and anti-NKp30 (Z25) and NKp46 (BAB281; Beckman Coulter).

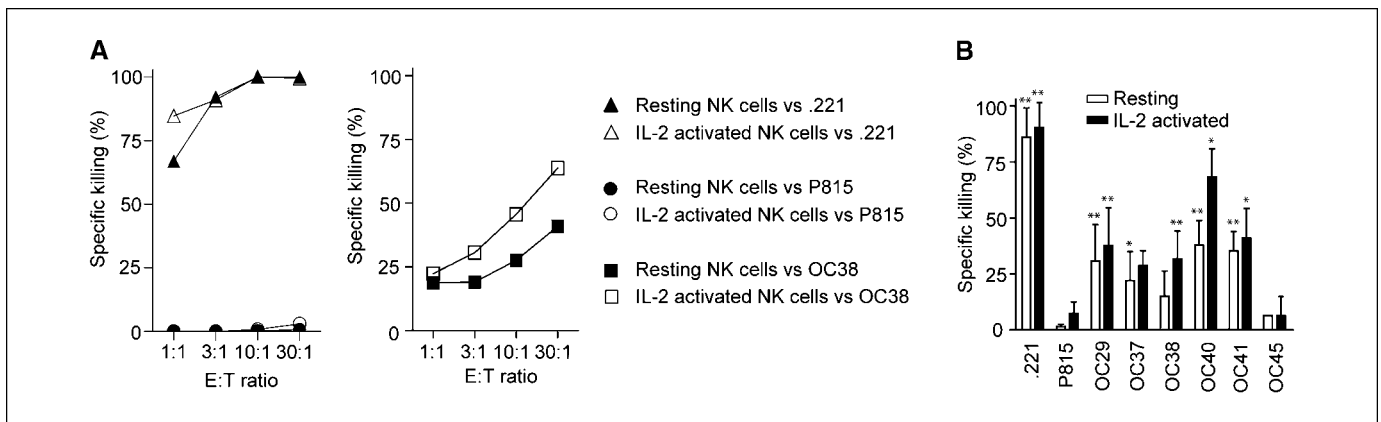
**Flow cytometry.** Cells were incubated with 1 µg of human IgG per 10<sup>5</sup> cells for 30 min on ice to block Fc receptors. Thereafter, cells were stained with nonconjugated primary mAbs followed by staining with a conjugated rat anti-mouse IgG1 mAb. For further staining with directly conjugated mAbs, free binding sites were blocked by incubating the cells with 5% mouse serum in PBS on ice for 30 min. Conjugated mAbs were added and incubated for 15 min on ice. Data were acquired on a CyAn ADP LX 9 color flow cytometer (DakoCytomation) and analyzed with FlowJo software (TreeStar, Ashland, OR).

**Analysis of NK cell degranulation.** NK cells were cocultured with target cells at a ratio of 1:1 in a final volume of 200 µL in round-bottomed 96-well plates at 37°C and 5% CO<sub>2</sub> for 6 h. Fluorochrome-conjugated anti-CD107a mAb or the corresponding IgG1 isotype control was added at the initiation of the assay. After 1 h of cocultivation, GolgiStop (monensin, Becton Dickinson) was added at a 1:100 dilution. Surface staining was done by incubating the cells with anti-CD3 and anti-CD56 mAbs for 15 min on ice. The cells were washed, resuspended in CellFix (Becton Dickinson), and analyzed by flow cytometry. For blocking experiments, NK cells were preincubated with 10 µg/mL of mAbs for 30 min at 4°C. HLA class I blockade was done by having A6-136 present in the assay at a dilution of 1:20.

**Flow cytometry-based cytotoxicity assays.** The CytoToxiLux-PLUS kit (OncoImmune, Gaithersburg, MD) was used to determine caspase-6 activity in the target cells. Resting or IL-2-activated NK cells were cocultured with target cells at a ratio of 30:1 in a final volume of 200 µL for 3 h at 37°C and 5% CO<sub>2</sub>. During the last 30 min of incubation, 75 µL of a cell-permeable substrate for caspase-6 were added. At the end of the assay, tumor cells were stained with the anti-EpCAM mAb. Staining was done on ice following Fc receptor blockade with IgG (1 µg/10<sup>5</sup> cells) to avoid antibody-dependent cellular cytotoxicity. In cocultures of tumor cells and fibroblasts, the latter were identified by staining with an anti-CD90 mAb. Control targets, lacking epithelial markers, were prelabeled with a membrane dye (TL4, OncoImmune). Cells were shielded from light and incubated for 10 min with 7-aminoactinomycin D (7-AAD; Becton Dickinson) at room temperature, and the samples were acquired immediately on the Cyan instrument.



**Figure 1.** Flow cytometry-based analysis of NK cell cytotoxicity. *A*, the NK-sensitive .221 and NK-resistant P815 cell lines were labeled with a fluorescent dye (TL4) and incubated for 3 h with or without resting NK cells. During the last 30 min of incubation, a cell-permeable fluorogenic substrate for caspase-6 was added. Apoptosis and cell death were monitored by determining caspase-6 activity and 7-AAD binding in TL4-labeled target cells. *B*, freshly isolated ovarian carcinoma cells were incubated with or without resting NK cells for 3 h. Caspase-6 activity and 7-AAD binding in EpCAM<sup>+</sup> ovarian carcinoma cells. Results are representative of 11 experiments.



**Figure 2.** Killing of freshly isolated ovarian carcinoma cells by resting NK cells. *A*, NK cell cytotoxicity was measured by flow cytometry. Titration of E:T ratio of resting (filled) and IL-2-activated (nonfilled) NK cells versus .221 cells (triangles, left), P815 cells (circles, left), and ovarian carcinoma cells (OC38; squares, right). Indicated values represent one of two independent experiments. *B*, NK cell killing of the indicated ovarian carcinoma cells at an E:T ratio of 30:1. .221 and P815 cells were used as positive and negative controls, respectively. Columns, mean of the specific killing obtained in 11 experiments; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Percentage specific cytotoxicity was calculated as follows:  $100 \times (\text{NK cell-induced apoptosis} - \text{spontaneous apoptosis}) / (100 - \text{spontaneous apoptosis})$ . For blocking experiments, NK cells were preincubated with 10  $\mu\text{g/mL}$  of mAbs for 30 min at 4°C. HLA class I blockade was done by having A6-136 present in the assay at a 1:20 dilution.

**Detection of granzyme B activity.** Resting or IL-2-activated NK cells were coincubated with target cells at an E:T ratio of 30:1 in a final volume of 200  $\mu\text{L}$  for 3 h at 37°C and 5%  $\text{CO}_2$ . During the last 30 min of incubation, 75  $\mu\text{L}$  of a cell-permeable substrate for granzyme B were added (GranToxiLux, Oncolmmune). To identify granzyme B activity in the target cells, ovarian carcinoma cells were stained with an anti-EpCAM mAb following incubation with NK cells, whereas .221 and P815 cells were prelabeled with a membrane dye (TL4). 7-AAD was added and cells were shielded from light and incubated for 10 min at room temperature before acquisition. When indicated, cell death was induced by exposing cells to medium containing 20% DMSO during 15 min before analysis of granzyme B activity.

**Statistics.** Statistical analyses were done using one-way ANOVA and Dunnett's test for multiple comparisons (GraphPad Software, Inc., San Diego, CA). Parametric (Pearson) calculations of correlations were done to analyze covariance.

## Results

**Lysis of freshly isolated tumor cells by resting NK cells.** To assess the ability of resting human NK cells to kill freshly isolated tumor cells, we used a flow cytometry-based assay to monitor induction of caspase-6 activity in target cells (12). Resting NK cells efficiently induced caspase-6 activity in the HLA class I-deficient .221 cell line (Fig. 1A). This was associated with significant cell death as assessed by 7-AAD binding to DNA in lysed target cells. In contrast, NK cells did not induce caspase-6 activity and cell death in the mouse mastocytoma cell line P815 (Fig. 1A). The killing of .221 cells by resting NK cells was in the range of that observed in classic  $^{51}\text{Cr}$  release assays, validating the use of the flow cytometry-based cytotoxicity assay (data not shown). For analysis of tumor cell killing in heterogeneous cell populations derived from freshly isolated peritoneal effusions from patients with ovarian carcinoma, we modified the protocol and stained for surface markers exclusively expressed on tumor cells immediately before flow cytometry analysis. Ovarian carcinoma cells were identified by forward/side scatter gating and by expression of the epithelial cell surface marker EpCAM (Fig. 1B). Resting allogeneic NK cells induced

activation of caspase-6 in freshly isolated EpCAM<sup>+</sup> ovarian carcinoma cells (Fig. 1B). Titration of E:T ratio revealed that cytotoxicity by resting NK cells was nearly in the range of that induced by short-term (18 h) IL-2-activated NK cells (Fig. 2A). A summary of 11 independent experiments revealed a high degree of NK cell-mediated lysis of five of six tested ovarian carcinoma (Fig. 2B). However, there was marked variability in the killing of the individual tumors. For example, the tumor OC40 was consistently killed at a high level by NK cells from several healthy donors, whereas OC45 was almost completely resistant to NK cells from all donors (Fig. 2B), suggesting that the variable tumor recognition was a nonrandom event mainly attributed to the tumor and not to the intrinsic NK cell activity of the donor.

**Freshly isolated ovarian carcinoma cells trigger NK cell degranulation.** Variability in the killing of different tumors may depend on their differential susceptibility to the effector functions of NK cells (i.e., different thresholds for apoptosis induction; ref. 13) but may also be a consequence of a varying capacity to trigger NK cell activation. To address this issue, we shifted focus from the target cell and monitored NK cell activation by analyzing the surface expression of CD107a on NK cells following contact with freshly isolated ovarian carcinoma cells (14, 15). Surface expression of CD107a correlates closely with degranulation and release of perforin and granzyme B by NK cells (5, 14). Approximately 20% of resting NK cells expressed CD107a on the cell surface on contact with the NK-sensitive .221 cell line (Fig. 3A). Freshly isolated ovarian carcinoma cells also triggered significant NK cell degranulation, albeit at lower levels compared with .221 cells (Fig. 3A). Short-term IL-2 activation of the NK cells led to enhanced NK cell degranulation in response to both .221 cells and freshly isolated ovarian carcinoma cells (Fig. 3A and B). Importantly, IL-2 alone did not induce CD107a expression by NK cells.

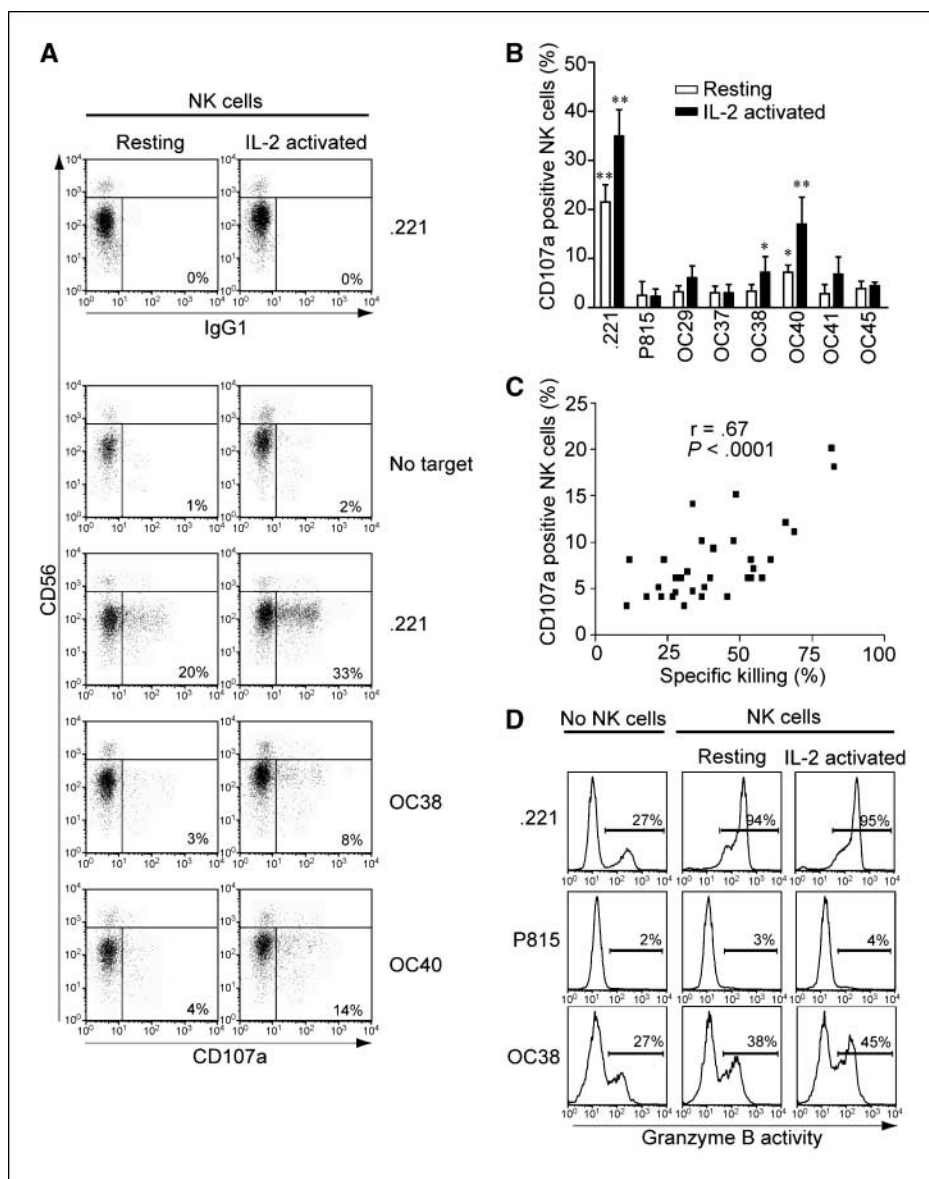
NK cell degranulation by resting and IL-2-activated NK cells following interaction with a panel of ovarian carcinoma was then investigated. The pooled data from 13 independent experiments showed that the induced NK cell degranulation was generally low but reproducible. The pattern of variability for NK cell degranulation was similar to that observed for the cytotoxic responses (Figs. 2B and 3B). Indeed, tumors that induced higher degrees of NK cell degranulation were also killed more efficiently, denoting a significant correlation between degranulation and cytotoxicity

(Fig. 3C). This outcome suggested that the low level of NK cell-mediated lysis of OC45 resulted from an inability of this tumor to activate NK cells rather than a general resistance to perforin and granzymes released by NK cells.

**Detection of granzyme B activity in freshly isolated tumor cells.** To investigate whether the rather low levels of degranulation by resting NK cells could account for the cytotoxic activity against the freshly isolated tumor cells, we monitored granzyme B activity in the tumor cells following coculture with NK cells. Incubation of resting NK cells with .221 cells led to significant granzyme B activity within the target cells (Fig. 3D). In contrast, incubation of NK cells with the resistant negative control target P815 did not lead to any detectable granzyme B activity. Importantly, degranulation by a small fraction of resting NK cells was sufficient to induce significant granzyme B activity in the ovarian carcinoma cells (OC38). These data indicate a major involvement of the perforin/granzyme B pathway in the recognition of ovarian carcinoma and strengthen the notion that activation and degra-

nulation of comparably few NK cells may result in lysis of a large proportion of tumor cells. To exclude the possibility that the increase in granzyme B activity in target cells following incubation with NK cells was due to nonspecific cleavage of the granzyme B substrate in preapoptotic or dead cells, we monitored granzyme B activity in .221 cells exposed to the toxic agent DMSO. Induction of cell death by DMSO did not lead to any detectable cleavage of the fluorogenic granzyme B substrate, indicating that release of granzyme B by NK cells was required (Supplementary Fig. S1).

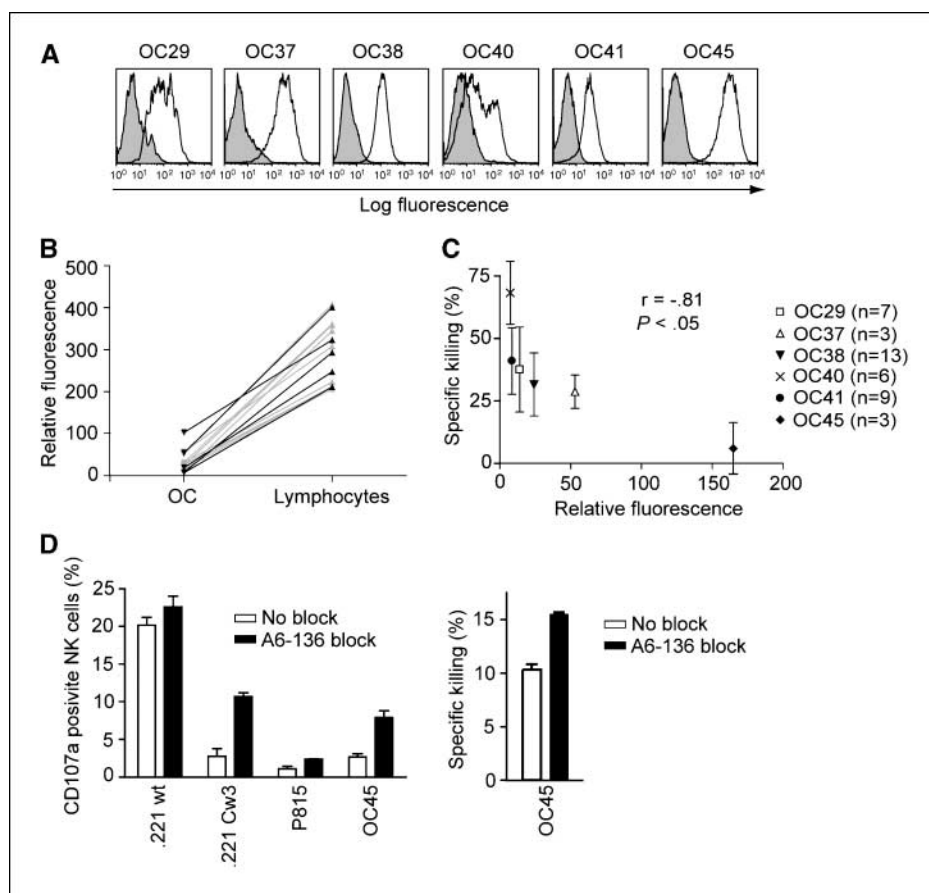
**Freshly isolated ovarian carcinoma cells express multiple ligands for activating NKRs.** NK cell cytotoxicity depends on a balance between signaling through inhibitory and activating receptors. Reduced levels of HLA class I expression may render target cells susceptible to NK cell-mediated lysis because these molecules deliver potent negative signaling via inhibitory NKRs (1, 16). The tumors included in this study displayed heterogeneous levels of HLA class I expression (Fig. 4A; ref. 9). The expression of



**Figure 3.** Degranulation of NK cells and granzyme B activity on interaction with freshly isolated ovarian carcinoma cells. *A*, resting and IL-2-activated NK cells were incubated for 6 h either alone or with the indicated target cells. Cells were stained with isotype control or anti-CD107a mAbs. Plots were gated on a forward scatter/side scatter lymphocyte gate. *Bottom right*, percentages of CD107a<sup>+</sup> CD56<sup>dim</sup> NK cells. *B*, NK cell degranulation on contact with the indicated ovarian carcinoma cells. .221 and P815 cells were used as positive and negative controls, respectively. *Columns*, mean percentage of CD107a<sup>+</sup> CD56<sup>dim</sup> NK cells from 13 experiments involving 29 donor-patient combinations; *bars*, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . *C*, correlation analysis of 31 simultaneous measurements of the percentage of CD107a<sup>+</sup> NK cells and percentage specific NK cell-mediated cytotoxicity on interaction with freshly isolated ovarian carcinoma cells. Pearson's test of covariance showed a significant correlation ( $P < 0.0001$ ) with the correlation coefficient  $r = 0.67$ . *D*, resting or IL-2-activated NK cells were incubated with .221 cells, P815 cells, or freshly isolated ovarian carcinoma cells (OC38) for 3 h. During the last 30 min of incubation, a cell-permeable fluorogenic substrate for granzyme B was added to the cocultures. Granzyme B activity within the indicated target cells following incubation with NK cells. Data are representative of two independent experiments.

Downloaded from <http://aacrjournals.org/cancerres/article-pdf/67/3/1317/12579372/1317.pdf> by guest on 13 April 2024

**Figure 4.** Inverse correlation between HLA class I surface expression on ovarian carcinoma cells and NK cell cytotoxicity. **A**, freshly isolated ovarian carcinoma cells were stained with anti-EpCAM mAb and either isotype control (*filled*) or anti-HLA class I mAb (W6/32; *line*). **B**, relative expression of HLA class I on ovarian carcinoma cells and autologous lymphocytes. *Black lines and triangles*, a total of 14 matched stainings is shown, of which 5 represent tumors investigated in detail in the current study. **C**, data on NK cell cytotoxicity toward the ovarian carcinoma cells are plotted against the relative fluorescence intensity of the anti-HLA class I stainings (geometric mean fluorescence intensity of the W6/32 staining/geometric mean fluorescence intensity of the isotype control). Pearson's test of covariance showed a significant correlation ( $P < 0.05$ ) with the correlation coefficient  $r = -0.81$ . **D**, effect of anti-HLA class I mAb (A6-136) on the degranulation (*left*) and cytotoxicity (*right*) by resting NK cells on contact with OC45 cells. .221wt, .221Cw3, and P815 cells were used to confirm the specific blocking effect of the anti-HLA class I mAb. *Columns*, mean of the results obtained from two independent donors.



HLA class I on tumors was on average 11% of that of autologous lymphocytes (Fig. 4B). Not unexpectedly, we observed an inverse correlation between the HLA class I expression and the degree of NK cell lysis of the ovarian carcinoma cells (Fig. 4C). The resistant ovarian carcinoma OC45 displayed the highest expression of HLA class I, whereas OC40 that was killed most efficiently by NK cells expressed the lowest levels of HLA class I. Thus, the variable expression of HLA class I by individual ovarian carcinoma provided a partial explanation for the observed variability in NK cell degranulation and cytotoxicity. Indeed, blockade of HLA class I led to increased degranulation and cytotoxicity by NK cells on contact with OC45 cells (Fig. 4D).

Activation of NK cells does not depend solely on the absence of HLA class I-mediated signaling through inhibitory NKR2s but requires positive signaling delivered through a wide array of activating NKR2s (3). Therefore, we examined the expression of several ligands for activating NKR2s, including the NKG2D ligands MICA, MICB, ULBP1, ULBP2, and ULBP3 (17, 18) as well as the recently identified ligands for DNAM-1, PVR (CD155), and Nectin-2 (CD112; ref. 19). One of six tumors analyzed expressed MICA and MICB (Fig. 5A). Expression of ULBP1, ULBP2, and ULBP3 was sparse, except for OC29 and OC40 cells, which exhibited high but heterogeneous expression of ULBP2, and OC37 and OC38 cells, which displayed weak expression of ULBP3 (Fig. 5A). Interestingly, PVR was ubiquitously expressed by ovarian carcinoma cells, whereas Nectin-2 was absent. K562 cells, used as positive controls in these stainings, expressed all ligands studied (Fig. 5A). These results showed that ovarian carcinoma cells express multiple

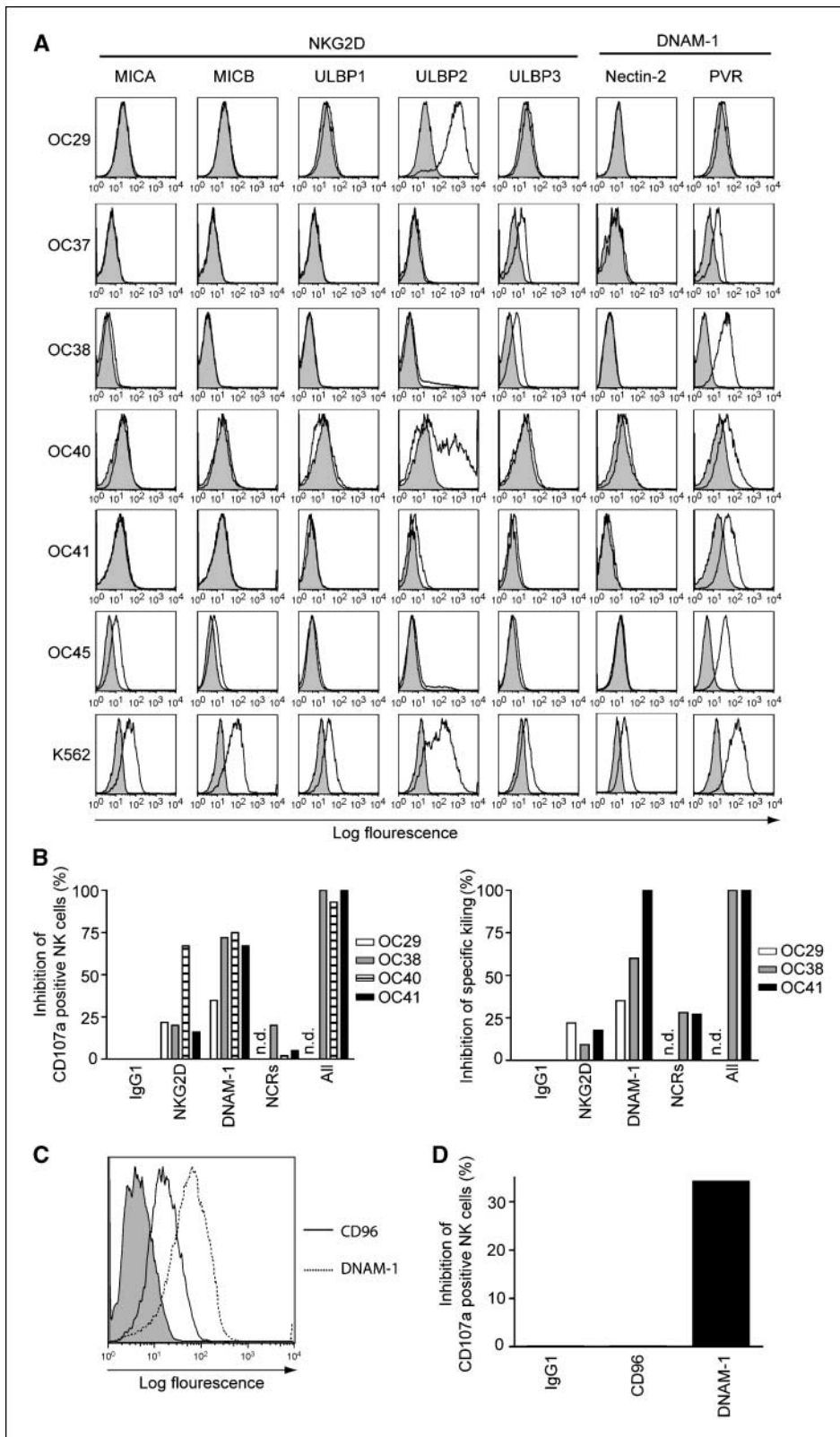
ligands for activating NKR2s, particularly the DNAM-1 ligand PVR, which is known to trigger antitumor responses (20, 21).

**Major role of DNAM-1/PVR interactions in NK cell recognition of freshly isolated ovarian carcinoma cells.** To determine the relative contribution of each receptor-ligand interaction monitored here, we did blocking experiments in which NK cells were preincubated with antibodies against NKG2D, NCR (NKP30 and NKP46), DNAM-1, or a combination of all four antibodies and then cocultured with freshly isolated ovarian carcinoma cells. Both degranulation (Fig. 5B, left) and cytotoxicity (Fig. 5B, right) were strongly inhibited by the anti-DNAM-1 antibody, indicating that DNAM-1/PVR interactions were critical for the activation of NK cells by most ovarian carcinoma. Recognition of OC29, which expressed the lowest levels of PVR, was less affected by DNAM-1 blockade (Fig. 5A and B). Moreover, NK cell degranulation on contact with OC40, which expressed both PVR and ULBP2, was substantially inhibited by both anti-DNAM-1 and anti-NKG2D antibodies (Fig. 5A). For some tumors, NK cell degranulation and cytotoxicity were weakly inhibited by the addition of anti-NCR antibodies, suggesting that the unknown ligands for these receptors were expressed by the ovarian carcinoma cells. Combining antibodies to all four NKR2s led to a complete block of degranulation and cytotoxicity, indicating that these receptor-ligand interactions complement each other in the activation of NK cells.

Recently, it was found that NK cell adhesion to target cells expressing PVR is promoted by CD96 (22, 23). To explore the possible involvement of CD96 in the recognition of ovarian

carcinoma, we monitored the expression of CD96 on resting NK cells. CD96 was expressed on resting NK cells but at lower levels compared with DNAM-1 (Fig. 5C). Presence of anti-CD96 mAb did not influence the recognition of freshly isolated ovarian carcinoma

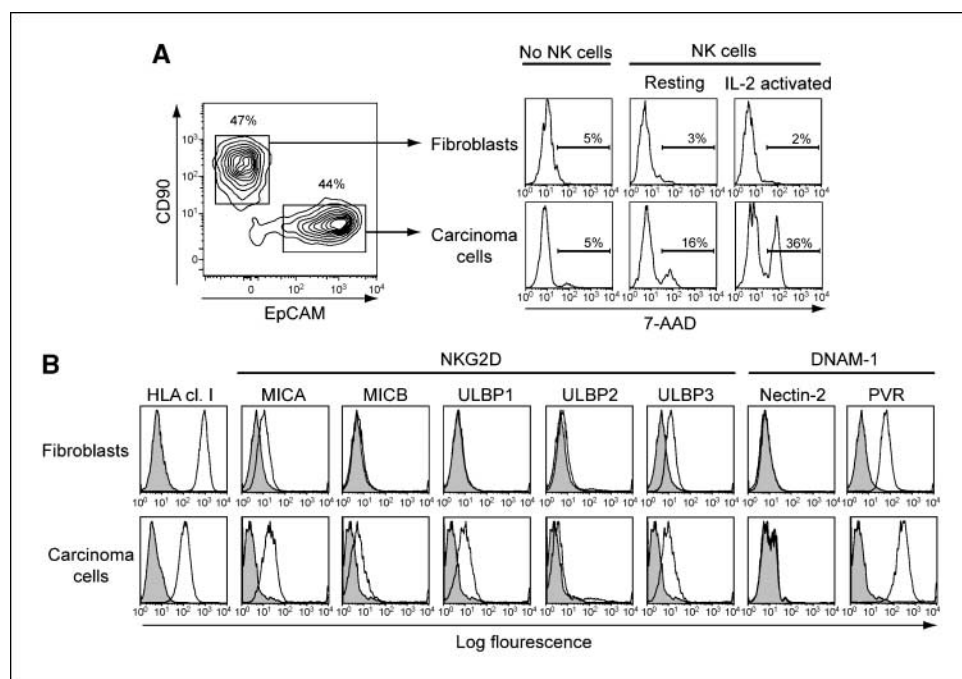
by resting NK cells, whereas anti-DNAM-1 significantly blocked NK cell degranulation (Fig. 5D) and cytotoxicity (data not shown). In agreement with previous studies, resting NK cells homogeneously expressed DNAM-1, NKG2D, NKp30, and NKp46 but lacked



**Figure 5.** Major role for DNAM-1/PVR interactions in the activation of NK cell degranulation and cytotoxicity. *A*, ovarian carcinoma cells were stained with isotype control (filled) or the indicated mAbs (solid line) to NKG2D and DNAM-1 ligands. The erythroleukemia cell line K562 was used as a positive control for all stainings. *B*, short-term IL-2-activated NK cells were analyzed for CD107a expression (left diagram) and cytotoxicity (right diagram) following incubation with freshly isolated ovarian carcinoma cells in the presence or absence of mAbs to the indicated NKRs. Percentage inhibition of degranulation and cytotoxicity. Data are representative of five independent experiments. *C*, resting NK cells were stained with 1  $\mu$ g/mL of primary nonconjugated mAbs to DNAM-1 or CD96 followed by an allophycocyanin-conjugated rat antimouse IgG1 mAb. *D*, resting NK cells were incubated with 10  $\mu$ g/mL of mouse IgG1, anti-DNAM-1, or anti-CD96. Percentage inhibition of degranulation of NK cells on contact with OC38.

Downloaded from <http://aacrjournals.org/cancerres/article-pdf/67/3/1322/93721317.pdf> by guest on 13 April 2024

**Figure 6.** Tumor specificity in NK cell recognition of ovarian carcinoma cells. **A**, short-term (2 weeks) cocultures of fibroblasts and ovarian carcinoma cells (OC38) from an individual patient were tested for susceptibility to killing by resting and IL-2-activated NK cells. Induction of cell death, as measured by 7-AAD binding, was monitored in fibroblasts (gated on CD90<sup>+</sup> cells) and ovarian carcinoma cells (gated on EpCAM<sup>+</sup> cells). Data are representative of two independent experiments. **B**, cocultures of fibroblasts and ovarian carcinoma cells (OC38) were stained with isotype control or mAbs to the indicated NKR ligands. The fibroblasts and ovarian carcinoma cells were identified by the expression of CD90 and EpCAM, respectively.



expression of Nkp44 (Supplementary Fig. S2; refs. 17, 19, 20, 24–26). Importantly, short-term IL-2 activation did not significantly alter the expression of activating receptors, except from a small increase in NKG2D expression, and failed to induce Nkp44 and CD96, known to be expressed at high levels after long-term culture in IL-2 (Supplementary Fig. S2; ref. 3). Taken together, these data show that DNAM-1 and PVR play major roles in the direct NK cell recognition of freshly isolated ovarian carcinoma cells with a complementary effect of other activating pathways determined by the ligands expressed on the individual tumors.

**Tumor specificity in NK cell recognition of ovarian carcinoma.** To ascertain that NK cells preferentially recognize tumor cells and spare normal tissues, we established short-term (2 weeks) cocultures of ovarian cancer cells and fibroblasts. Resting NK cells were able to discriminate tumor cells from the patient-derived fibroblasts (Fig. 6A), suggesting a significant degree of tumor specificity by the NK cells. The short-term culture period affected the expression of several of the ligands for activating NK receptors on the ovarian carcinoma cells, leading to a more pronounced expression of MICA, ULBP1, ULBP3, and PVR (Figs. 5 and 6B). However, under these experimental conditions, the fibroblasts displayed higher expression of HLA class I and lower expression levels of several ligands for activating receptors compared with the tumor cells (Fig. 6B). This differential expression of HLA class I and activating NKR ligands might enable NK cells to discriminate tumor cells from the surrounding normal tissue, thereby offering a therapeutic window in which NK cells can operate without causing unwanted damage to normal tissues.

## Discussion

NK cells were named for their capacity to recognize tumor cells without the need for prior sensitization (27), and their capacity to kill murine and human tumor cells is well documented. However, the requirements for recognition of freshly isolated human tumors by resting/nonactivated NK cells have not or only sparsely been

explored. A better understanding of the capacity of resting NK cells to kill primary human tumors seems important because there is hope to develop novel treatment strategies against cancer based on infusion of nonmanipulated or short-term IL-2-activated allogeneic NK cells. Moreover, the repertoire of donor-derived NK cells that contribute to the GVL effect in allogeneic stem cell transplantation (8) is likely to share phenotypic properties with fresh, nonactivated NK cells.

For the current analysis, we used a flow cytometry-based cytotoxicity assay to monitor caspase-6 activation and cell death in freshly isolated ovarian carcinoma cells (12). We have shown that freshly isolated ovarian carcinoma cells are susceptible to resting NK cells derived from allogeneic healthy donors. Overall cytotoxicity reflects the net sum of multiple discrete events, including NK cell activation, release of cytolytic granules, and susceptibility of a tumor to NK cell effector pathways. Therefore, we shifted focus from the fate of the target cells to the activation of effector cells and monitored the capacity of freshly isolated ovarian carcinoma cells to trigger degranulation by resting and short-term IL-2-activated NK cells. To address whether the variability in cytotoxic responses reflected a reduced NK cell activation, we compared the percentage of CD107a<sup>+</sup> NK cells with their cytotoxic activity. This analysis revealed a significant correlation between degranulation and cytotoxicity, which would indicate that the variability in cytotoxicity and the reduced killing of some tumors depended on a failure to induce NK cell degranulation.

NK cell killing of ovarian carcinoma cells correlated inversely with the level of HLA class I on the cell surface of the tumors. One of the tumors, OC45, expressed comparably high levels of HLA class I and was more resistant to resting NK cells. Antibody-mediated masking of HLA class I led to increased NK cell degranulation on contact with OC45. The blockade of HLA class I interactions also resulted in higher induction of cell death of OC45 cells. Altogether, these results strengthen the interpretation that HLA class I expression is a major constraint for the activation of resting NK cells by freshly isolated ovarian carcinoma. However,

one might have expected a higher degree of killing of OC45 in light of the relatively profound increase in NK cell degranulation following HLA class I blockade. The discrepancy between the level of NK cell activation and target cell lysis may depend on target cell resistance to apoptosis commonly observed in tumor cells (13). An alternative explanation could be that the target cells express low levels of adhesion molecules leading to nonpolarized degranulation. This has previously been described for NK cell activation in the absence of leukocyte functional antigen-1/intercellular adhesion molecule-1 interactions (5). Indeed, OC45 expressed relatively low levels of CD54, which could favor this explanation (data not shown).

Ample evidence indicates that the susceptibility of several tumor types to NK cell cytotoxicity may be predicted by their NKR ligand repertoire (28–30). To gain insights into the activating pathways that were involved in the activation of resting NK cells by freshly isolated ovarian carcinoma, we analyzed their NKR ligand repertoire. Ovarian carcinoma displayed ubiquitous expression of PVR, whereas the expression of NKG2D ligands was more variable. However, the differential susceptibility to resting NK cells among the ovarian carcinoma studied could not be explained by the expression pattern of these ligands because even the resistant tumor expressed both PVR and MICA. Although the expression of NKR ligands could not predict the NK cell susceptibility, their presence seemed critical for NK cell recognition. Hence, masking the corresponding activating receptors on the NK cells inhibited NK cell recognition. Accordingly, recognition of ovarian carcinoma cells was mainly dependent on DNAM-1, although NKG2D influenced recognition of tumors that expressed any of the NKG2D ligands. Indeed, NK cell degranulation in response to an ovarian carcinoma, expressing both ULBP2 and PVR, was blocked by both anti-NKG2D and anti-DNAM-1 antibodies. Masking NCR partially inhibited NK cell recognition of some tumors, indicating that unknown ligands for these receptors may be expressed by ovarian carcinoma cells. The contribution of DNAM-1 and NKG2D to NK cell activation was complementary rather than synergistic, which is in line with a recent study where these receptors were shown to synergize with Nkp46 and 2B4 but not with each other (4). We found no obvious synergies between NKG2D or DNAM-1 and Nkp46, but these results are difficult to evaluate in the absence of defined ligands for Nkp46. Synergy with 2B4 could not be tested because ovarian carcinoma cells were negative for the 2B4 ligand CD48. Because CD96 is another receptor that was recently shown to contribute to NK cell target cell adhesion through interaction with PVR, we explored the involvement of CD96 in the NK cell recognition of ovarian carcinoma (22, 31). The expression of CD96 was not induced by overnight incubation in IL-2, indicating that the induction of CD96 on activation, as observed by others, requires longer culture times or other types of stimulation (22, 23). Blocking experiments showed that CD96 did not contribute to the activation of resting NK cells by PVR-expressing ovarian carcinoma cells. It cannot be excluded that CD96 may influence the recognition of ovarian carcinoma cells by polyclonal IL-2-activated NK cell cultures or *in vivo* in the inflammatory environment surrounding the tumor in peritoneal ascites.

Because NK cell tolerance to self is partially preserved by expression of inhibitory receptors for self HLA class I molecules, autologous NK cells could theoretically be used to target HLA class I<sup>low</sup> ovarian carcinoma cells. However, NK cells derived from cancer patients often display reduced function as a consequence of tumor-induced immune suppression (32). Indeed, there is substantial evidence of poor *ex vivo* activity of NK cells derived from ovarian

carcinoma patients against the prototype NK cell target K562 and autologous tumors (33–36). Therefore, it may be more advantageous to use allogeneic NK cells from healthy donors. In agreement with the “missing self” hypothesis (16), allogeneic NK cells may be specifically triggered in a KIR/HLA-mismatched situation (i.e., when a patient lacks one or more of the HLA motifs for inhibitory KIR expressed on donor NK cells; ref. 8). However, KIR/HLA mismatch may be of less importance when faced with HLA class I<sup>low</sup> tumors as exemplified by the current study of ovarian carcinoma cells. Moreover, the use of resting NK cell populations with a clonally distributed KIR repertoire may limit the beneficial effect of a KIR/HLA mismatch because a majority of the NK cells, even in a KIR/HLA-mismatched donor, express KIR and/or other inhibitory receptors (NKG2A and LILR-B1) for ligands on the patient’s tumor cells. The role of KIR/HLA mismatch in the recognition of HLA class I<sup>low</sup> ovarian carcinoma by resting allogeneic NK cells is currently under investigation in our laboratory.

A major concern for the use of allogeneic NK cells in settings of immunotherapy is that they could cause a tissue-damaging “graft-versus-host” (GVH) reaction. However, no GVH disease (GVHD) was observed in several recent clinical trials involving infusion of allogeneic NK cells to patients with malignant disease (6, 7). Moreover, no increased GVHD was observed despite significant GVL effects in KIR ligand-mismatched stem cell transplantation for AML. In part, this was explained by clearance of dendritic cells known to initiate T-cell-mediated GVHD disease (8, 37), but the lack of GVHD also implies a capacity of NK cells to discriminate tumor cells from normal tissues. Our data indicate that the recognition of ovarian carcinoma cells by allogeneic NK cells may involve a certain degree of tumor specificity. We showed that NK cells lysed ovarian carcinoma cells but spared fibroblasts derived from the same patient. The fibroblasts expressed higher levels of HLA class I and lower levels of activating NKR ligands. Although PVR was also expressed by fibroblasts, the specific reduction of HLA class I expression and simultaneous expression of ligands for activating NKRs by tumor cells may lower the thresholds for NK cell activation, thereby leading to tumor-specific targeting by allogeneic NK cells. It remains elusive if ovarian carcinoma cells within the ovary itself display similar expression of PVR and low levels of HLA class I, making them susceptible to the recognition of NK cells. Further studies are required to address whether the expression of NK receptor ligands is modulated during tumor progression and/or by the tumor environment (i.e., peritoneal ascites).

In conclusion, our data show that resting NK cells readily recognize and kill freshly isolated ovarian carcinoma cells. Recognition was dependent on DNAM-1 signaling with complementary contributions of NKG2D and NCR receptors. The susceptibility of ovarian carcinoma to NK cell-mediated cytotoxicity suggests that this tumor type may be a suitable candidate for immunotherapy based on adoptive transfer of allogeneic NK cells.

## Acknowledgments

Received 6/21/2006; revised 10/30/2006; accepted 12/7/2006.

**Grant support:** Swedish Foundation for Strategic Research, Swedish Research Council, Swedish Cancer Society, Swedish Society for Medical Research, Swedish Children’s Cancer Foundation, Cancer Society of Stockholm, Karolinska Institutet, Royal Academy of Sciences, Tobias Foundation, NIH grant no. CA102280, Dutch Cancer Society, and Novartis Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.



## References

1. Long EO, Rajagopalan S. HLA class I recognition by killer cell Ig-like receptors. *Semin Immunol* 2000;12:101–8.
2. Lopez-Botet M, Llano M, Navarro F, Bellon T. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol* 2000;12:109–19.
3. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005;23:225–74.
4. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 2006;107:159–66.
5. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med* 2005;202:1001–12.
6. Miller JS, Soignier Y, Panoskaltis-Mortari A, et al. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005;105:3051–7.
7. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia* 2004;18:1835–8.
8. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002;295:2097–100.
9. Norell H, Carlsten M, Ohlum T, et al. Frequent loss of HLA-A2 expression in metastasizing ovarian carcinoma associated with genomic haplotype loss and HLA-A2-restricted HER-2/neu-specific immunity. *Cancer Res* 2006;66:6387–94.
10. Vitale M, Pelusi G, Taroni B, et al. HLA class I antigen down-regulation in primary ovary carcinoma lesions: association with disease stage. *Clin Cancer Res* 2005;11:67–72.
11. Malmberg KJ, Levitsky V, Norell H, et al. IFN- $\gamma$  protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J Clin Invest* 2002;110:1515–23.
12. Liu L, Chahroudi A, Silvestri G, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nat Med* 2002;8:185–9.
13. Malmberg KJ, Ljunggren HG. Escape from immune- and nonimmune-mediated tumor surveillance. *Semin Cancer Biol* 2006;16:16–31.
14. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods* 2004;294:15–22.
15. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8<sup>+</sup> T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281:65–78.
16. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 1990;11:237–44.
17. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999;285:727–9.
18. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 2001;14:123–33.
19. Bottino C, Castriconi R, Pende D, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* 2003;198:557–67.
20. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* 2002;17:19–29.
21. Pende D, Bottino C, Castriconi R, et al. PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis. *Mol Immunol* 2005;42:463–9.
22. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J Immunol* 2004;172:3994–8.
23. Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. *J Immunol* 1992;148:2600–8.
24. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med* 1999;190:1505–16.
25. Pessino A, Sivori S, Bottino C, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med* 1998;188:953–60.
26. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* 1998;187:2065–72.
27. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 1975;5:112–7.
28. Carbone E, Neri P, Mesuraca M, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* 2005;105:251–8.
29. Castriconi R, Dondero A, Corrias MV, et al. Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res* 2004;64:9180–4.
30. Pende D, Spaggiari GM, Marcenaro S, et al. Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). *Blood* 2005;105:2066–73.
31. Fuchs A, Colonna M. The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. *Semin Cancer Biol* 2006;16:359–66.
32. Malmberg KJ. Effective immunotherapy against cancer: a question of overcoming immune suppression and immune escape? *Cancer Immunol Immunother* 2004;53:879–92.
33. Lai P, Rabinowich H, Crowley-Nowick PA, Bell MC, Mantovani G, Whiteside TL. Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma. *Clin Cancer Res* 1996;2:161–73.
34. Lotzova E, Savary CA, Freedman R, Bowen JM. Natural immunity against ovarian tumors. *Comp Immunol Microbiol Infect Dis* 1986;9:269–75.
35. Lotzova E, Savary CA, Freedman RS, Edwards CL, Wharton JT. Recombinant IL-2-activated NK cells mediate LAK activity against ovarian cancer. *Int J Cancer* 1988;42:225–31.
36. Roszkowski PI, Hyc A, Malejczyk J. Natural killer cell activity in patients with ovarian tumors and uterine myomas. *Eur J Gynaecol Oncol* 1993;14 Suppl:114–7.
37. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 1999;285:412–5.