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Natural Killer Cell-Mediated Killing of Freshly Isolated Neuroblastoma Cells: Critical Role of DNAX Accessory Molecule-1–Poliovirus Receptor Interaction

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ABSTRACT

In the present study, we assessed the susceptibility of freshly isolated neuroblastoma cells to killing mediated by normal human natural killer (NK) cells and analyzed the receptor–ligand interactions that regulate this event. We show that killing of freshly isolated neuroblasts, similar to neuroblastoma cell lines, involves NKp46 and NKp30 (natural cytotoxicity receptors). However, freshly isolated neuroblasts were generally more resistant to NK-mediated lysis than conventional neuroblastoma cell lines. Moreover, a significant heterogeneity in susceptibility to lysis existed among neuroblastomas derived from different patients. Remarkably, susceptibility to lysis directly correlated with the surface expression, on neuroblasts, of poliovirus receptor [PVR (CD155)], a ligand for the DNAX accessory molecule-1 [DNAM-1 (CD226)] triggering receptor expressed by NK cells. Indeed, PVR-expressing neuroblastomas were efficiently killed by NK cells. Moreover, monoclonal antibody-mediated masking of either DNAM-1 (on NK cells) or PVR (on neuroblasts) resulted in strong inhibition of tumor cell lysis. Thus, assessment of the PVR surface levels may represent a novel useful criterion to predict the susceptibility/resistance of neuroblastomas to NK-mediated killing.

INTRODUCTION

Natural killer (NK) cells display cytolytic activity against virus-infected cells and a wide variety of tumors of different histotype (1). The function of NK cells is controlled by an array of different receptors that, upon engagement by specific target cell ligands, may either induce or suppress the process of killing (2, 3). The inhibitory receptors include the HLA class I-specific killer immunoglobulin-like receptors and CD94/NKG2A heterodimers (4–7). The interaction of HLA molecules expressed on normal cells with HLA class I-specific NK receptors results in protection of these cells from NK-mediated lysis. On the other hand, NK cells can kill target cells in the absence of interaction between inhibitory receptors and HLA class I molecules. This occurs in the case of allogeneic cells or HLA-defective targets such as tumor or virally infected cells (8). Target cell killing depends on the engagement of specific activating receptors and coreceptors expressed at the NK cell surface with surface ligands expressed on target cells. NKp46, NKp30, and NKp44 molecules, collectively termed natural cytotoxicity receptors [NCRs (9)], as well as NKG2D (10) appear to play a predominant role in the process of NK-mediated killing of most target cells, whereas 2B4, NTB-A, CD59, and NKp80 primarily function as coreceptors (11–13). The majority are orphan receptors because their cellular ligands are still elusive. The exceptions are represented by NKG2D, which has been shown to recognize major histocompatibility complex class I-related chain molecules A/B (MICA/B) and UL16-binding proteins (ULBPs) (14), 2B4, which reacts with CD48 (15), and NTB-A, which is characterized by homophilic recognition (16). Another surface molecule that has been shown to participate in the induction phase of NK cell activation is DNAX accessory molecule-1 [DNAM-1 (CD226)], which is expressed by virtually all human NK cells, T cells, and monocytes. Recently, it has been shown that DNAM-1 recognizes poliovirus receptor [PVR (CD155)] and Nectin-2 (CD112; ref. 17), two closely related molecules belonging to the nectin family. PVR and Nectin-2 are widely expressed on normal neuronal, epithelial, endothelial, and fibroblastic cells (18) and at high density on tumors of different histotype (17).

Notably, most studies on the molecular mechanisms enabling human NK cells to kill tumors have been based on the use, as target cells, of *in vitro* cultured cell lines, whereas the ability to kill freshly isolated tumor cells has been investigated in only a few cases (19). This is primarily due to difficulties in purifying tumor cells from surrounding normal tissues. Also, in the case of neuroblastoma, most available data were obtained by the assessment of NK-mediated killing of various tumor cell lines. In particular, we showed that NK cells display strong cytolytic activity against cultured neuroblasts (including ACN and SK-N-BE) and that NCR, but not NKG2D, are involved in the mechanisms leading to killing (20, 21). In line with this observation, neuroblastoma cell lines did not express surface ligands for the NKG2D receptor.

In this study, we analyzed the susceptibility to NK-mediated killing of highly purified neuroblastoma cells isolated from patients at stage 4 of the disease. While confirming the role of NCR in the process of killing, we show that freshly isolated neuroblastoma cells may or may not express PVR, a ligand recognized by the DNAM-1 receptor. Importantly, expression of PVR was confined to neuroblastoma cells that displayed the highest susceptibility to NK-mediated killing. In line with these results, a critical role of DNAM-1–PVR interaction in the NK-mediated killing of neuroblastoma cells could be demonstrated.

MATERIALS AND METHODS

Monoclonal Antibodies. 5B14 monoclonal antibody [mAb (IgM, anti–B7-H3); ref. 22], c218 (IgG1, anti-CD56), A6136 (IgM, anti-HLA class I), KRA236 (IgG1, anti–DNAM-1; ref. 17), KL247 (IgM, anti-NKp46), F252 (IgM, anti-NKp30), M5A10 (IgG1, anti-PVR; ref. 17), L14 (IgG2A, anti–Nectin-2; ref. 17), M7E22 [IgG1, anti–intercellular adhesion molecule (ICAM)-1], and DF305 [IgM, anti-lymphocyte function-associated antigen (LFA)-3] were produced in our laboratory. MAbs anti-GD_2 (14.G2a, IgG2A) and anti-CD45 (HI30, IgG1) were purchased from BD Biosciences PharMingen (San Diego, CA) and Caltag Laboratories (Burlingame, CA), respectively.

Bone Marrow Aspirates and Neuroblastoma Purification. After informed consent was obtained, bone marrow was aspirated from iliac crests with a 1.8-gauge needle from children diagnosed with neuroblastoma and admitted to the Hematology-Oncology Division of the G. Gaslini Institute between January 2003 and January 2004. Diagnosis and staging were performed according to the International Neuroblastoma Staging System (23). Neuroblastoma cells were purified from bone marrow aspirates by CD45

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depletion for Enrichment of Circulating Tumor Cells KIT (RosetteSep; Stem Cell Technologies, Vancouver, British Columbia, Canada).

The investigation was performed after approval by the Gaslini Institute institutional review board.

Polyclonal Natural Killer Cells. NK cells were purified using the Human NK Cell Enrichment Cocktail-RosetteSep (Stem Cell Technologies) and cultured on irradiated feeder cells in the presence of 100 units/mL recombinant interleukin-2 (Proleukin; Chiron Corp., Emeryville, CA) and 1.5 ng/mL phytohemagglutinin (Gibco Ltd., Paisley, United Kingdom) to obtain polyclonal NK cell populations (17).

Cytolytic Activity and Flow Cytofluorometric Analysis. NK cells were tested for cytolytic activity against the indicated target cells in a 4-h ^{51}Cr release assay as described previously (17). The concentrations of the various mAbs added for masking experiments were 10 μg/mL. The effector to target (E/T) ratios are indicated in the text.

For one-color cytofluorometric analysis (FACSCalibur; Becton Dickinson, Mountain View, CA) cells were stained with the appropriate mAbs followed by phycoerythrin (PE)-conjugated isotype-specific goat antimouse second reagent (Southern Biotechnology Associated, Birmingham, AL; ref. 17).

RESULTS

Analysis of Neuroblastoma Cells as Target for Natural Killer Cell-Mediated Cytotoxicity. Previous studies indicated that metastatic neuroblastoma cells infiltrating the bone marrow in patients at stage 4 can be distinguished from hematopoietic cell precursors on the basis of their CD45-negative, GD_2-positive, and B7-H3 (B7 homologue 3)–positive surface phenotype (22–26). To purify neuroblastoma cells, bone marrow aspirates obtained from children diagnosed with neuroblastoma at stage 4 (see Material and Methods) were depleted of both CD45-positive hematopoietic cells and erythroid cells. The resulting cell populations displayed typical neuroblastoma morphologic hallmarks (data not shown; ref. 22) and homogeneously displayed a surface phenotype consistent with that of neuroblastoma cells. Thus, >95% of the cells were characterized by a lack of CD45 and by expression of the B7-H3 and GD_2 neuroblastoma-associated surface markers and CD56 molecules (Fig. 1; refs. 22–26). Purified neuroblastoma cells were isolated from bone marrow aspirates of eight different patients. In no instance could we detect neuroblastoma cells lacking the above-mentioned cell markers. Among the *in vitro* established neuroblastoma cell lines, HTLA230 (Fig. 1), Gi-LI-N, and SH-SY5Y (data not shown), similar to the purified neuroblastoma cells, displayed the CD45−/GD_2+/B7-H3+/CD56+ surface phenotype. On the contrary, other neuroblastoma cell lines, including SK-N-BE (Fig. 1) and GIMEN (data not shown), lacked GD_2 and CD56 molecules. The various samples of metastatic neuroblastoma cells were also analyzed for expression of HLA class I molecules (Fig. 1; data not shown). Consistently, freshly isolated neuroblasts either lacked or expressed negligible amounts of these molecules. This result is consistent with those regarding the majority of *in vitro* cultured neuroblastoma cell lines (Fig. 1; ref. 20), all of which lacked surface HLA class I molecules, with the exception of the ACN cell line.

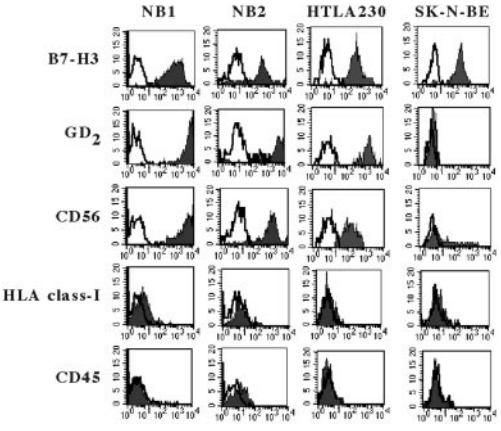

Fig. 1. Comparative cytofluorometric analysis of freshly isolated or long-term cultured neuroblastoma cells. Neuroblastoma cells (NB1 and NB2) purified from bone marrow aspirates in children affected by stage 4 neuroblastoma or the representative HTLA230 and SK-N-BE neuroblastoma cell lines were analyzed by one-color fluorescence and cytofluorometric analysis with mAbs to the indicated molecules, followed by a PE-conjugated goat antimouse isotype-specific second reagent. *White profiles* refer to cells incubated with the second reagent only.

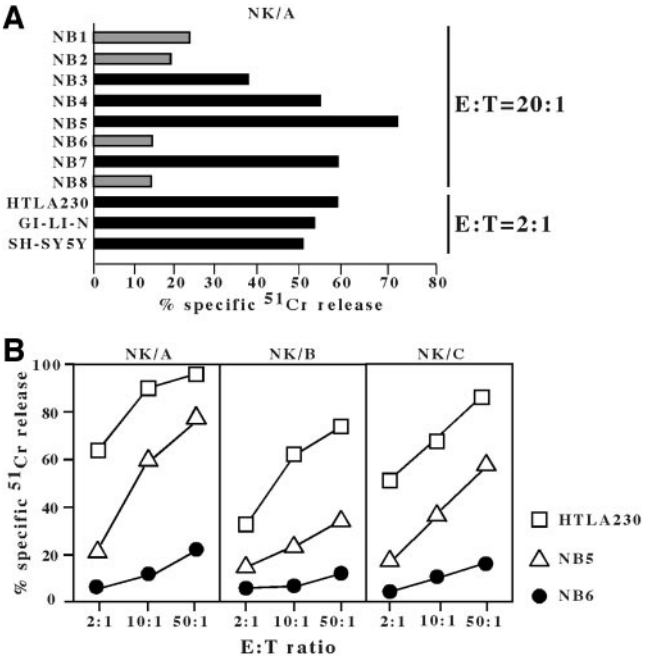

Fig. 2. Neuroblastoma cells derived from different patients are heterogeneous in their susceptibility to NK-mediated killing. *A*. A representative polyclonal NK cell population from a normal donor (*NK/A*) was analyzed for cytolytic activity against freshly isolated neuroblastoma cells from eight different patients or against the indicated cell lines. The E/T cell ratio used is indicated. The results are representative of triplicate experiments; the SD of the mean of the triplicates was <5%. *B*. Three polyclonal NK cell populations from normal unrelated donors (*NK/A*, *NK/B*, and *NK/C*) were analyzed for cytolytic activity against freshly isolated neuroblastoma cells from representative patients 5 (△) and 6 (●) or against the HTLA230 cell line (□) at various E/T ratios. The results are representative of triplicate experiments; the SD of the mean of the triplicates was <5%.

The defective expression of HLA class I molecules represents a favorable condition for allowing NK-mediated lysis (2, 8). Thus, we evaluated the susceptibility of fresh neuroblastoma cells to lysis mediated by interleukin-2 cultured polyclonal NK cells. As shown in Fig. 2, freshly isolated neuroblasts were generally more resistant to NK-mediated cytolytic activity than representative neuroblastoma cell lines (~10-fold difference). Moreover, a significant heterogeneity in susceptibility to lysis existed among neuroblasts derived from different patients. Thus, as shown in Fig. 2, neuroblasts derived from patients 3, 4, 5, and 7 were killed more efficiently by NK cells than those derived from patients 1, 2, 6, and 8. Similar results were obtained using polyclonal NK cells from three different healthy donors as effectors. It can be seen that although NK cells from these donors displayed substantial differences in their cytolytic activity, freshly isolated neuroblastoma cells were consistently less susceptible to lysis as compared with cultured neuroblastoma cell lines (Fig. 2*B*).

As expected, in view of the low expression or absence of HLA class I molecules, NK-mediated lysis of fresh neuroblasts could not be increased in the presence of anti-HLA class I mAb (data not shown).

Analysis of the Activating Natural Killer Receptors that Are Involved in Killing of Neuroblasts. NK-mediated lysis of various tumor cell lines of different origin is due to the engagement of

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multiple triggering receptors by ligands expressed at the target cell surface (13). However, the role of individual NK receptors may differ, depending on the expression levels of their specific ligands. In this context, NKG2D has been shown to play a predominant role in NK-mediated killing of T-cell leukemia (21). On the other hand, both NCR and DNAM-1 were responsible for recognition and killing of neuroblastoma cell lines including HTLA230 (17). Here we analyzed which activating interaction(s) is crucial for NK-mediated killing of freshly isolated neuroblastoma cells. To this end, neuroblastoma cells isolated from different stage 4 patients were analyzed for their susceptibility to lysis by different polyclonal NK cell populations from healthy donors in either the absence or presence of mAbs specific for an individual triggering receptor (Fig. 3). Interestingly, killing of neuroblasts derived from patients 4, 5 (Fig. 3), 3, and 7 (data not shown) appeared to be highly dependent on the engagement of DNAM-1. Thus, mAb-mediated masking of DNAM-1 resulted in inhibition of lysis comparable with that resulting after blocking of NCR. A virtual abrogation of lysis occurred after simultaneous mAb-mediated masking of both DNAM-1 and NCR. This indicates that both DNAM-1 and NCR contribute to NK cell activation against neuroblastoma cells. Notably, tumor cells from these patients belonged to the group of neuroblastoma displaying high susceptibility to NK-mediated killing (see Fig. 2). On the contrary, mAb-mediated blocking of DNAM-1 had virtually no effect on lysis of tumor cells derived from patients 6 (Fig. 3), 1, 2, and 8 (data not shown), whereas a significant inhibitory effect was obtained after blocking of NCR. Thus, DNAM-1 only marginally contributed to lysis of those neuroblastoma cells characterized by poor susceptibility to NK-mediated killing.

Taken together, these data indicated that DNAM-1–ligand interactions may be critically involved in NK-mediated killing of some freshly isolated neuroblasts. In addition, DNAM-1 involvement in cytolysis correlated with the susceptibility or resistance of a given neuroblastoma to NK-mediated killing.

Differential Expression of the DNAM-1 Ligands PVR and Nectin-2 in Freshly Isolated Neuroblastoma Cells. The above-mentioned results suggested that the susceptibility of neuroblastoma cells to NK-mediated lysis might depend on the expression levels of the ligands (17) specifically recognized by DNAM-1. To verify this possibility, we analyzed neuroblastoma cells isolated from the various patients for surface expression of PVR (CD155) and Nectin-2 (CD112). As shown in Fig. 4, PVR was expressed in neuroblasts derived from patients 3, 4, 5, and 7, whereas it was virtually absent in those isolated from patients 1, 2, 6, and 8. In addition, among PVR-positive neuroblastomas, a correlation existed between the levels of expression of PVR and susceptibility to NK-mediated lysis. Indeed, neuroblasts from patient 5, which expressed the highest levels of PVR, displayed the highest susceptibility to lysis. On the other hand, PVR-negative neuroblasts consistently displayed poor susceptibility to lysis (see Fig. 2). Expression of Nectin-2 could be detected only in neuroblasts isolated from patients 2, 5, and 7. However, the expression of Nectin-2 did not render tumor cells from patient 2 susceptible to NK-mediated lysis. This finding suggests the lack of correlation between expression of Nectin-2 and susceptibility to lysis.

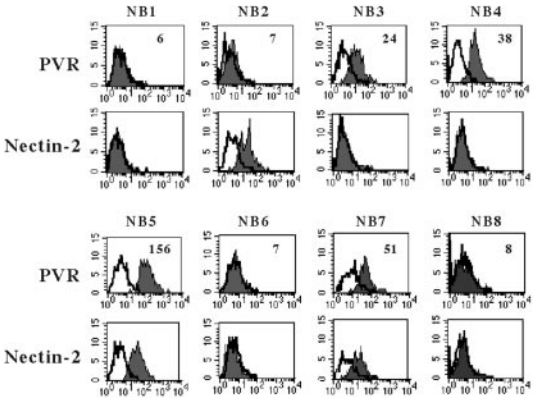

Fig. 4. Analysis of PVR and Nectin-2 surface expression in freshly isolated neuroblastoma cells. Purified tumor cells from bone marrow aspirates derived from eight different neuroblastoma patients were analyzed by one-color fluorescence and cytofluorometric analysis for expression of PVR and Nectin-2. PE-conjugated goat antimouse isotype-specific was used as second reagent. *White profiles* refer to cells incubated with the second reagent only. *Numbers* in the *top right* of *panels* represent mean fluorescence intensities.

It is noteworthy that none of the neuroblastoma cells tested expressed major adhesion molecules including ICAM-1 and LFA-3 (data not shown). Thus, the differences in susceptibility to NK-mediated lysis cannot be consequent to differences in the expression of these adhesion molecules.

The Interaction between DNAM-1 and PVR Plays a Crucial Role in Natural Killer-Mediated Lysis of Freshly Isolated Neuroblastoma Cells. The above-mentioned data suggested that differences among neuroblastomas in their susceptibility to NK-mediated killing could primarily be related to expression of PVR on neuroblasts. In order to further substantiate this hypothesis, we analyzed the effect of mAb-mediated masking of PVR or Nectin-2 on the NK-mediated lysis of different neuroblastoma cells. As shown in Fig. 5, killing of PVR-positive tumor cells (see representative patient 5) was significantly inhibited by mAb-mediated masking of PVR. The magnitude of inhibition was comparable with that induced by masking of the DNAM-1 receptor. As expected, killing of PVR-negative neuroblasts (see representative patient 6) was unaffected by anti-PVR mAb. Finally, mAb-mediated masking of Nectin-2, expressed by neuroblast of patients 5 (Fig. 5) and 7 (data not shown), did not result in any inhibitory effect on NK-mediated lysis. Similar results were obtained using polyclonal NK cells from two other normal donors as effectors (data not shown). Taken together, these data indicate that the interaction between DNAM-1 and PVR plays a crucial role in NK-mediated killing of freshly isolated neuroblastoma cells.

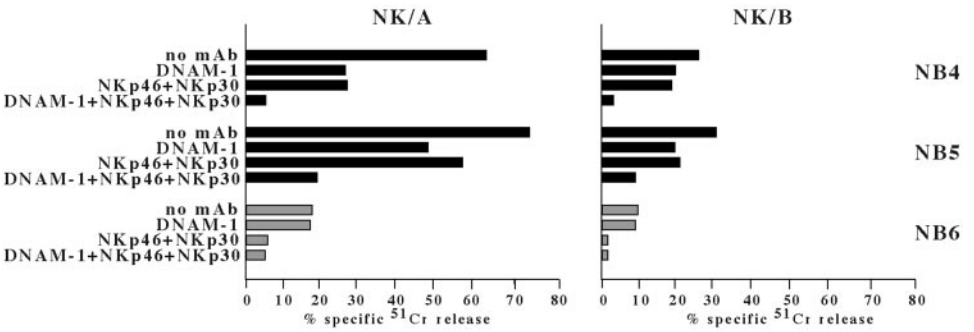

Fig. 3. Involvement of NK receptors in NK-mediated killing of freshly isolated neuroblastoma cells. Two representative polyclonal NK cell populations from normal donors (*NK/A* and *NK/B*) were analyzed for cytolytic activity against freshly isolated neuroblastoma cells from patients 4, 5, and 6 in either the absence of mAb or the presence of mAbs to the indicated molecules, used either alone or in combination. The E/T cell ratio used was 20:1. The results are representative of triplicate experiments; the SD of the mean of the triplicates was <5%.

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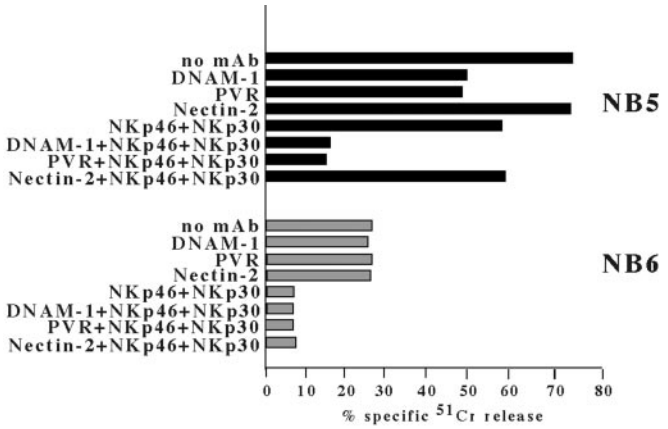

Fig. 5. Analysis of the role of PVR and Nectin-2 in NK-mediated killing of freshly isolated neuroblastoma cells. A representative polyclonal NK cell population from a normal donor was analyzed for cytolytic activity against freshly isolated neuroblastoma cells from patients 5 and 6 in either the absence or presence of mAbs to the indicated molecules, used either alone or in combination. The E/T cell ratio used was 20:1. The results are representative of three independent experiments; the SD of the mean of the triplicates was <5%.

DISCUSSION

In the present study, we provide evidence that freshly isolated neuroblastoma cells are susceptible to NK-mediated lysis. More importantly, we show that a key role in the lytic process is played by PVR, a molecule expressed at the tumor cell surface that is recognized by the DNAM-1 receptor. We analyzed highly purified, fresh neuroblastoma cells isolated from bone marrow aspirates (22). As compared with cultured neuroblastoma cell lines (20), freshly isolated neuroblasts were generally more resistant to lysis. Remarkably, a certain degree of variability existed among different tumors. In particular, we show that tumor cells displaying maximal susceptibility to lysis were characterized by high surface expression of PVR. This molecule was recently recognized as a ligand for DNAM-1, a surface receptor mediating NK cell activation and tumor cell killing (17). In line with these findings, we now demonstrate that mAb-mediated disruption of DNAM-1–PVR interactions inhibits NK-mediated killing of neuroblasts. Thus, recognition of PVR by DNAM-1 critically contributes to the NK-mediated killing of a fraction of neuroblastoma cells and suggests that simple analysis of the expression of PVR by these tumors may be predictive of their susceptibility to NK-mediated killing.

In previous studies (20), we showed that NCRs but not NKG2D were involved in NK-mediated killing of neuroblastoma cell lines. We now confirm the relevant role of NKp30 and NKp46 in the mechanisms of NK cell triggering leading to lysis of freshly isolated neuroblasts. Indeed, in all neuroblastomas analyzed, the simultaneous mAb-mediated blocking of NKp46 and NKp30 resulted in inhibition of lysis. However, additional receptor–ligand interactions could cooperate to induce optimal NK cell activation and killing of neuroblastomas. This hypothesis was analyzed by mAb-mediated blocking experiments. Using this experimental approach, we provide clear evidence that recognition of PVR (on tumor cells) by DNAM-1 (on NK cells) represents a molecular interaction essential for the induction of optimal NK-mediated killing of a subset of neuroblastomas. In line with this observation, we found that NK cells preferentially kill neuroblasts expressing PVR, whereas they display low levels of cytotoxicity against tumor cells lacking PVR. Among PVR-positive neuroblastomas, it has also been possible to directly correlate the levels of PVR expression to the magnitude of NK-mediated lysis. Along this line, a remarkable observation is that in the cases analyzed thus far, PVR-negative neuroblasts were isolated from patients in relapse, whereas PVR-expressing neuroblasts were from children at the onset of the disease. Although this observation needs to be confirmed in a larger number of patients, it may suggest that tumor cells in advanced stages of the disease down-regulate the surface expression of an important ligand for NK-mediated activation against neuroblastoma. This may be viewed as a mechanism of tumor escape from NK cell-mediated killing (8). Although data are not shown, we also evaluated the functional capability of polyclonal NK cell populations derived from different neuroblastoma patients. In all patients, both the expression of various triggering NK receptors and cytolytic activity were comparable with that of NK cells derived from healthy, age-matched children. Thus, in contrast to previously described findings in acute myeloid leukemia patients (19), NK cells from neuroblastoma patients appear to display normal functional capabilities. This is in line with the hypothesis that down-regulation of relevant tumor ligands for triggering NK receptors might play a role in favoring the process of tumor progression (19, 21). Moreover, other mechanisms of tumor escape might be based on the expression of molecules that protect tumor cells from NK cell-mediated attack. Along this line, we recently showed that B7-H3 molecules are expressed on all stage 4 neuroblastoma patients and can be used as a cell surface marker for specific recognition of neuroblastoma cells in bone marrow infiltrates. Remarkably, the engagement of B7-H3 by still undefined inhibitory NK receptors could partially down-regulate NK cell cytotoxicity (22).

It is conceivable that the absence of major adhesion molecules (including ICAM-1 and LFA-3) in the neuroblastomas analyzed (both at onset and during relapse) allowed us to better appreciate the contribution of PVR in determining susceptibility to NK-mediated killing. In this regard, DNAM-1 is known to be involved not only in NK cell activation but also in cell–cell adhesion (18, 27). This suggests that the DNAM-1–PVR interaction may also provide a mechanism allowing NK cells to adhere to neuroblasts.

It is noteworthy that expression of Nectin-2 does not seem to be relevant in determining the magnitude of NK cell-mediated killing of neuroblasts. Previous studies, however, demonstrated that both PVR and Nectin-2 trigger NK cell cytotoxicity on specific engagement of DNAM-1 (17). A possible interpretation of our present data might be that the surface density of Nectin-2 required to elicit responses via DNAM-1 may be higher than that expressed in neuroblasts from patients 2, 5, and 7. Along this line, we have shown previously (17) that DNAM-1 binds strongly to PVR but less efficiently to Nectin-2. These data, together with the finding that, in some cases, neuroblasts expressed PVR and Nectin-2 at similar surface densities (for example, see NB7 in Fig. 4, whose lysis was inhibited by anti-PVR but not by anti-Nectin-2 mAb), support the concept that the surface density of PVR required for DNAM-1–dependent NK cell activation may be lower than that of Nectin-2. Very recently, CD96, also termed "Tactile", has been identified as a novel receptor for PVR (28). Thus, it cannot be ruled out that also CD96 may contribute to recognition of PVR on tumor cells.

Our present study provides evidence that neuroblastoma cells are heterogeneous in their susceptibility to NK-mediated killing. Moreover, it highlights the critical role of PVR–DNAM-1 interaction in the mechanisms of NK cell triggering leading to tumor cell killing. Thus, assessment of PVR expression may allow prediction of whether or not a given neuroblastoma will be susceptible to NK cell-mediated attack. This may be relevant when attempting novel NK cell-based immunotherapeutic protocols (for example, in protocols based on myeloablative chemotherapy followed by autologous stem cell transplantation and infusion of autologous activated NK cells). Indeed, in the case of PVR-positive

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NK-susceptible neuroblastomas, NK cells could play a role in the control of residual tumor cells and possibly in their elimination.

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