NK Cells Use NKG2D to Recognize a Mouse Renal Cancer (Renca), yet Require Intercellular Adhesion Molecule-1 Expression on the Tumor Cells for Optimal Perforin-Dependent Effector Function[1]

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The NKG2D receptor on NK cells can recognize a variety of ligands on the tumor cell surface. Using a mouse renal cancer (Renca), we show that NKG2D recognition by NK cells was crucial for their ability to limit tumor metastases in vivo in both liver and lungs using perforin-dependent effector mechanisms. However, for the R331 cell line established from Renca, NKG2D recognition and perforin-dependent lysis played no role in controlling liver metastases. R331 cells were also more resistant to perforin-dependent lysis by NK cells in vitro. We therefore used these phenotypic differences between Renca and R331 to further investigate the crucial receptor:ligand interactions required for triggering lytic effector functions of NK cells. Reconstitution of R331 cells with ICAM-1, but not Rae-1γ, restored NKG2D-mediated, perforin-dependent lysis. Interestingly, R331 cells were efficiently lysed by NK cells using death ligand-mediated apoptosis. This death ligand-mediated killing did not depend on NKG2D recognition of its ligands on tumor cells. This result suggests that the intracellular signaling in NK cells required for perforin and death ligand-mediated lysis of tumor target cell are quite distinct, and activation of both of these antitumor lytic effector functions of NK cells could improve therapeutic benefits for certain tumors. *The Journal of Immunology,* **2006, 177: 2575–2583.**

Natural killer cells are innate immune effector cells that play a critical role for host defense against viruses and tumors (1). The development of tumor metastases in various organs can be limited by the local activity of NK cells (2, 3). NK cells can use multiple mechanisms to promote tumor destruction in vivo. These include perforin-dependent (4) or death ligand-mediated NK cell cytotoxicity (5, 6). In addition, the production by NK cells of IFN-γ may also impede tumor development (7, 8). The relative importance of each of these effector mechanisms for tumor destruction may depend on the activation status of the NK cells, the inherent biological characteristics of the tumor cells themselves, as well as the local tissue environment.

NK recognition of tumor cells is complex. NK cells can recognize self by binding to class I MHC molecules. This recognition results in inhibitory signaling by many members of the Ly49 family of proteins (9). In addition, recognition of the nonclassical MHC molecule Qa-1 by the CD94-NKG2 heterodimers may result in inhibitory signaling (10, 11). However, both of these NK cell receptor families also contain members that stimulate NK cell effector functions following contact with tumor cells. Recently, there has been much interest in the NKG2D molecule as a stimulatory receptor on NK cells (12). A variety of unrelated ligands have been identified that bind to NKG2D. In the mouse, these ligands include H60 and the Rae1 family (α,β,γ,δ,ε) (13, 14) and the more recently identified MULT1 (15). Transfection of these NKG2D ligands (NKG2DL)[4] can sensitize tumor cells to NK cell-mediated lysis in vitro (15–18). Furthermore, a number of studies have indicated that recognition by NK cells of NKG2DL on tumor cells can limit tumor development in vivo (13, 16), and that NKG2D signaling predominantly engaged the perforin-mediated effector pathway (19). This effector function of NK cells could be amplified in vivo by administration of various cytokine treatments such as IL-2, IL-12 (20), and IL-21 (21). NKG2D/NKG2DL recognition by NK cells was pivotal for the antimetastatic activity of these cytokines that promoted perforin-mediated cytotoxicity. By contrast, the antitumor effects of IL-18 were dependent on the expression of Fas by the tumor and Fas ligand (FasL) on NK cells, yet did not depend on NKG2D-NKG2DL recognition (20). NKG2D/NKG2DL recognition and perforin-dependent effector function has

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[4] Abbreviations used in this paper: NKG2DL, NKG2D ligand; FasL, Fas ligand; WT, wild type; anti-asGM1, anti-asialoGM1; CMA, concanamycin A.

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also been implicated in protecting mice against the spontaneous development of sarcomas following injection with methylcholanthrene (22). Strikingly, the perforin-mediated cytolysis was important not only in controlling tumor development but also in determining the ultimate tumor phenotype. Sarcomas developed more rapidly in pfp$^{-/-}$ mice exposed to methylcholanthrene than in wild-type (WT) mice. In addition, the sarcomas isolated from pfp$^{-/-}$ mice often expressed Rae-1 ligands, whereas those from WT mice did not. These sarcomas with high cell surface expression of Rae-1 were rejected by WT but not pfp$^{-/-}$ mice, presumably due to immunoediting. Nonetheless, due to the complexity of NK cell recognition, there remains the possibility that other receptors on NK cells may have crucial roles in modifying either recognition or the intracellular signaling following contact with tumor target cells. We have recently described a clone of Renca (R331) that exhibited a very different phenotype in vivo from that of the parental Renca cell line (23). Numbers of liver metastases of R331 in vivo were limited by death receptor-death ligand interactions. In this study, we show that perforin-mediated lysis by NK cells had no significant effect on the development of R331 liver metastases, in striking contrast to the metastases of parental Renca cells. We have therefore used these differences between Renca and R331 tumor target cells to further investigate the crucial receptor-ligand interactions required by NK cells to trigger both perforin and death ligand-mediated cytolysis.

Materials and Methods

Mice

Specific pathogen-free BALB/c mice were obtained from the Animal Production Area, National Cancer Institute (NCI; Frederick, MD) or The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at the Peter MacCallum Cancer Centre (East Melbourne, Australia). BALB/c RAG-1$^{-/-}$, BALB/c perforin-deficient (BALB/c pfp$^{-/-}$), BALB/c TRAIL-deficient (BALB/c TRAIL$^{-/-}$), BALB/c IFN-γ-deficient (BALB/c IFN-$\gamma^{-/-}$), BALB/c mice doubly deficient for perforin and IFN-γ (BALB/c pfp$^{-/-}$IFN-$\gamma^{-/-}$) and doubly deficient for perforin and TRAIL (BALB/c pfp$^{-/-}$TRAIL$^{-/-}$). BALB/c RAG-2$^{-/-}$ mice were purchased from Taconic Farms and bred at the NCI-Frederick. Mice were maintained in a dedicated pathogen-free environment and used between 6 and 12 wk of age. Animal care was provided in accordance with the procedures outlined in A Guide for the Care and Use of Laboratory Animals (National Institutes of Health; publication no. 86-23, 1985).

Target cells and reagents

Renca is a BALB/c-derived renal adenocarcinoma cell line. R331, provided by Dr. R. Wiltrout (NCI-Frederick, Frederick, MD), is a clone that was originally established from the parental Renca cell line by limiting dilution. All of the cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1× nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), 10 mM HEPES, and 5×10^{-5} M 2-ME (pH 7.4) (complete medium). Recombinant human IL-2 was provided by Hoffman-La Roche, and recombinant mouse IL-18 was purchased from BioSource International. The hamster mAbs A10 and C7 to NKG2D were generated as described previously (24). Rat mAbs (CX1) raised against mouse Rae-1γ or a pan-Rae-1 Ab (25) were provided by Dr. L. Lanier (University of California, San Francisco, CA). All experiments were performed at least three times with similar findings.

NK cell isolation

Mice received an i.p. injection of 100 μg of poly(I:C) (obtained from Sigma-Aldrich) on day 0, and spleen cells or liver mononuclear cells were isolated 18 h later as described previously (26, 27). For NK depletion, WT or gene-targeted mice were injected with 20 μg of polyclonal rabbit anti-asialoGM1 (anti-asGM1) Ab (Wako Chemicals) on days −1 and 0. We recently reported that the i.v. injection of cDNA expression vectors in large volumes (hydrodynamic shear) can produce high serum levels of the specific protein. i.v. injection of 4 μg of a cDNA expression plasmid for IL-2 in a total volume of 1.6 ml of saline causes the dramatic recruitment of large numbers of NK cells to the liver (28). Therefore, mononuclear cells from livers from BALB/c mice injected 3–4 days previously with the IL-2 plasmid were isolated as described previously. For further purification of lymphoid subsets, lymphoid cells were incubated for 20 min with a mixture of conjugated Abs CD3-biotin, CD19-biotin, and CD24-biotin to specifically label T and B cells and various other leukocytes excluding NK cells (BD Biosciences). Cells that had bound Abs were depleted using the MACS separation system as recommended by the manufacturer (Miltenyi Biotec). The purity of the NK cell-enriched fraction that eluted from the MACS columns were assessed by FACS analysis using the mAbs CD5-Per-CP (T cells) and DX5-PE (NK cells). Purity of enriched liver NK cells from mice treated with cDNA for IL-2 was usually >90%.

Cytotoxicity assays

Target cells were labeled with ^{111}In-labeled oxine ([^{111}In] Ox; Amersham Health, Medi-Physics) or $Na_2{}^{51}CrO_4$ (New England Nuclear) as described previously (29). Labeled cells (1×10^4) were then incubated with effector cells at various ratios for 16 to 18 h at 37°C in a final volume of 200 μl. After incubation, supernatants were harvested and counted on a gamma counter. Specific killing (percentage of cytotoxicity) was calculated as follows: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. All groups were run in triplicate, and all experiments were performed three or more times with similar findings. In some experiments, IL-2 (500 U/ml) and IL-18 (10 ng/ml) were added to the cytotoxicity assay. Also included in some experiments at 20 μg/ml were neutralizing mAbs to NKG2D (A10 or C7), CD18 (BD Biosciences), and mouse FasL (MFL-1) or mouse TRAIL (N2B2) (provided by Dr. H. Yagita, Juntendo University, Tokyo, Japan). In some experiments, concanamycin A (CMA; Sigma-Aldrich) at 50 ng/ml was added to the effector cells for 3 h before addition of the targets cells to inhibit perforin-mediated lysis.

Flow cytometric analysis

For analysis of NKGD binding, NKG2D tetramers were used to stain cells as described previously (30). For staining with the rat monoclonal CX1, or the pan-Rae 1 Ab, cells were incubated with 0.5 μg of Ab per 10^6 cells followed by appropriate goat anti-rat IgG2-PE-conjugated secondary Ab at 0.5 μg/10^6 cells. For staining of ICAM-1, a PE-labeled mAb (BD Biosciences) was diluted in and used in accordance with instructions of the manufacturers. Flow cytometry analysis was performed on a FACScan using CellQuest (BD Biosciences) or FACS Express 3 (De Novo software) software. The stained cells were analyzed on a FACScan, and data were processed by the CellQuest program (BD Biosciences).

Generation of R331 cells expressing Rae-1γ and ICAM-1

A cDNA expression vector for mouse ICAM-1 provided by Dr. B. Blazar (University of Minnesota, Minneapolis, MN) was transfected into R331 cells using standard procedures, and a vector with no cDNA insert (R331-VC) was used as a control. A cDNA expression vector for mouse Rae-1γ was provided by Dr. L. Lanier (University of California, San Francisco, CA). Stable transfectants of R331-Rae-1γ or controls transfected using the vector lacking the cDNA insert (R331-puroVC) were generated as described previously (13). R331 double transfectants containing both ICAM-1 and Rae-1γ and appropriate vector controls (R331-VC-puroVC) were also generated. After 2–3 wk selection, R331 transfectants were stained with appropriate Abs and sorted on a BD FACSAria sorter (BD Biosciences Immunocytometry Systems) using a 100-μm nozzle and a low pressure set up (Coherent Sapphire solid state laser at 488 nm and 13-m W of power).

Tumor growth and experimental metastasis assay in vivo

BALB/c WT or gene-targeted mice were injected intrasplenically with 5×10^4 Renca or R331 cells and i.v. with 5×10^3 Renca cells (to produce lung metastases) as described previously (8). Mice were euthanized 14 days after tumor inoculation, and liver (after intrasplenic) or lung (after i.v.) metastases were quantified with the aid of a dissecting microscope. In some experiments, mice were depleted of NK cells by i.p. injection with 100 μg of anti-asGM1 on days 0, 1, and 7 relative to tumor inoculation. Some groups of BALB/c mice were treated with either hamster anti-mouse NKG2D mAb (C7) or hamster control Ig mAb by i.p. injection of 250 μg of Ab on days 0, 1, 7, and 8 after tumor inoculation. All experiments were performed at least twice with 5–10 mice per experimental group.

Statistical analysis

The significance of difference in number of metastasis between experimental groups was determined by the unpaired Mann-Whitney *U* test. Two-sided p values of <0.05 are considered significant. For cytotoxicity experiments, significance between experimental groups was determined using the Student's t test, and p values of <0.005 were considered significant.

Results

NKG2D and perforin-dependent restriction of Renca but not R331 metastases in vivo

We have previously characterized a clone of Renca designated R331 that differs dramatically from the parental tumor in its increased susceptibility to death receptor-mediated apoptosis (23). We therefore compared the development of Renca and R331 metastases in vivo in various gene-targeted mice in the presence or absence of blocking Abs to NKG2D (Table I). NK cells play a crucial role in controlling lung metastases of Renca cells because treatment of mice with anti-asGM1 significantly enhances numbers of metastases in the lungs from 30 to 130 in WT BALB/c mice. In BALB/c pfp$^{-/-}$ mice, numbers of lung metastases were also significantly increased above WT. Increases in metastases were also noted in IFN-$\gamma^{-/-}$ mice, as previously reported for other experimental tumor models (7). Interestingly, numbers of Renca lung metastases did not differ between WT and TRAIL$^{-/-}$ mice. Because numbers of metastases in pfp$^{-/-}$ $\times$ IFN-$\gamma^{-/-}$ mice (136) were essentially identical with numbers in mice treated with anti-asGM1 (130), it seems that ability of NK cells to restrict lung metastases of Renca is primarily dependent on both perforin and IFN-γ. Treatment of WT mice with anti-NKG2D significantly increased numbers of lung metastases from 30 to 58, which was a similar increase to that observed in pfp$^{-/-}$ mice or IFN-$\gamma^{-/-}$ mice. However, on treatment of pfp$^{-/-}$ mice with anti-NKG2D, no further increase in numbers of metastases was observed. In contrast, in IFN-$\gamma^{-/-}$ mice treatment with anti-NKG2D further increased metastases numbers from 58 to 120. Also, treatment of TRAIL$^{-/-}$ mice treatment with anti-NKG2D significantly increased metastases from 29 to 49. This increase suggests that the blocking of NKG2D in vivo only abrogates the beneficial effects of perforin in reducing numbers of Renca lung metastases, yet has no effects on the control of metastases by IFN-γ or TRAIL. The anti-NKG2D injections resulted in the blocking of NK activity, but the NK cells were not depleted by this treatment (data not shown).

The data obtained for Renca liver metastases were similar to those observed for lung metastases. Following treatment with anti-asGM1, metastases numbers increase from WT levels of 70 to 221. However, restriction of liver metastases is a little more complex. Perforin, IFN-γ, and TRAIL all played some role because numbers of metastases in pfp$^{-/-}$ (151), IFN-$\gamma^{-/-}$ (156), and TRAIL$^{-/-}$ mice (140) were all significantly elevated as compared with WT. The treatment of mice with anti-NKG2D increased numbers of metastases from 70 to 117 in WT mice. Anti-NKG2D treatment also significantly increased numbers of Renca liver metastases in IFN-$\gamma^{-/-}$ and TRAIL$^{-/-}$ mice, yet had no effect on metastases in pfp$^{-/-}$ mice. Once again this increase suggests that the major effects in vivo on blocking interactions between the NKG2D on NK cells and its ligands (NKGD2L) on tumor cells are on the perforin-dependent lytic pathway, and this interaction has little influence on the antitumor effects mediated by IFN-γ or TRAIL. Indeed, no detectable production of IFN-γ was observed following coincubation of purified NK cells with Renca in vitro (data not shown). This result suggests that NKG2D signaling in NK cells following contact with Renca was not sufficient to trigger production of detectable IFN-γ.

In direct contrast to Renca, the number of R331 liver metastases was dependent on the expression of TRAIL by local effector cells. TRAIL$^{-/-}$ mice exhibited 199 liver metastases, an almost 10-fold increase over numbers of metastases in WT mice (20). No further increases in numbers of R331 liver metastases were observed in either WT mice, pfp$^{-/-}$, or TRAIL$^{-/-}$ following treatment with anti-NKG2D (Fig. 1). The fact that there are no changes in numbers of R331 liver metastases in vivo in either the absence of perforin or following anti-NKG2D treatment suggests that perforin-dependent lysis by NK cells plays no obvious beneficial role in restricting R331 liver metastases in vivo. Also, TRAIL effector function of liver NK cells seems independent of the NKG2D-NKG2DL molecular interaction. Because NKG2D-mediated, perforin-dependent effects did not seem to influence R331 liver metastases in vivo, we decided to further investigate the molecular basis of this difference between Renca and R331 cells.

Table I. *NKG2D recognition reduces Renca lung and liver metastases by NK perforin-dependent effector function*

		Lung			Liver		
Mouse Strains	Treatment	Number of metastases ($\pm$SE)[a]	p value vs IgG[b]	p value vs WT mice[c]	Number of metastases ($\pm$SE)[a]	p value vs IgG[b]	p value vs WT mice[c]
WT	Control IgG	30 (4)			70 (7)		
	Anti-NKG2D	**58 (6)**	<0.05		**117 (6)**	<0.05	
pfp$^{-/-}$	Control of IgG	62 (4)		<0.05	151 (4)		
	Anti-NKG2D	64 (4)			158 (7)		<0.05
IFN$\gamma^{-/-}$	Control IgG	58 (5)		<0.05	156 (5)		
	Anti-NKG2D	**120 (7)**	<0.05		**194 (5)**	<0.05	
TRAIL$^{-/-}$	Control IgG	29 (6)			140 (4)		<0.05
	Anti-NKG2D	**49 (6)**	<0.05		**187 (8)**	<0.05	
pfp$^{-/-}$Trail$^{-/-}$	Control IgG	66 (3)		<0.05	208 (6)		<0.05
	Anti-NKG2D	61 (7)			211 (8)		
pfp$^{-/-}$IFN$\gamma^{-/-}$	Control IgG	136 (6)		<0.05	221 (6)		<0.05
	Anti-NKG2D	138 (4)			221 (13)		
WT (anti-asGM1-treated)	Control IgG	130 (5)		<0.05	221 (9)		<0.05
	Anti-NKG2D	135 (3)			223 (6)		

[a] Numbers of metastases $\pm$ SE.
[b] Significance of differences in metastases comparing mice treated with control IgG or anti-NKG2D. Significant increases in metastases numbers also bolded.
[c] Significance of differences between metastases comparing WT mice and other gene-targeted strains or WT mice treated with anti-asGM1.

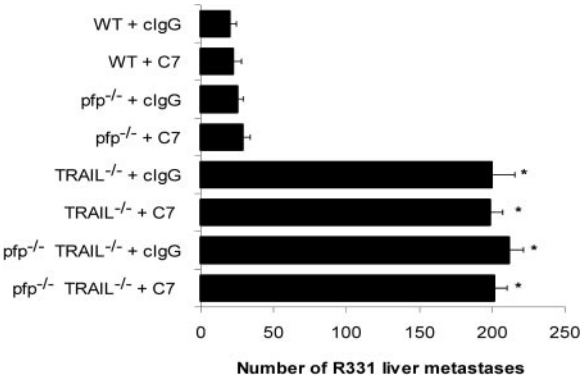

FIGURE 1. NKG2D recognition has no effect on R331 liver metastases. Groups of 5–10 WT, pfp$^{-/-}$, TRAIL$^{-/-}$, and pfp$^{-/-}$/TRAIL$^{-/-}$ mice were inoculated intrasplenically with 5×10^4 R331 tumor cells on day 0. Some groups of mice, as indicated, received anti-NKG2D (C7) or control Ig (250 μg i.p.) on days 0, 1, 7, and 8. The livers were removed at day 14, and the metastatic nodules were quantified. Data are recorded as the mean numbers of metastases ± SE, with significant differences from WT mice treated with control IgG as defined by the Mann-Whitney *U* test; ∗, $p < 0.05$.

Different mechanisms for NK lysis of Renca and R331 cells in vitro

Because there was a dramatic difference in the role of NKG2D-NKG2DL in limiting Renca but not R331 liver metastases in vivo, we examined the lytic potential of liver NK cells isolated from various gene-targeted mice. Renca cells were significantly more susceptible to NK-mediated lysis than R331cells when using effector cells isolated from the livers of mice treated with poly(I:C) (Fig. 2). All lytic activity against either Renca or R331 was abolished by anti-asGM1 treatment of the mice, suggesting it was NK mediated. However, the molecular mechanisms used to lyse Renca or R331 differed substantially. Concerning lysis of Renca, lytic activity of liver NK cells from pfp$^{-/-}$ mice was much reduced compared with WT NK cells, whereas the lysis of R331 by WT and pfp$^{-/-}$ NK cells was equivalent (Fig. 2*A*). By contrast, TRAIL$^{-/-}$ NK cells had only a slightly reduced ability to lyse Renca than WT NK, yet were unable to lyse R331 targets. Similar findings were observed using Rag 2$^{-/-}$ liver NK cells as effectors. Lysis of Renca was significantly higher than that of R331 (Fig. 2*B*). Blocking Abs to TRAIL abolished the lysis of R331 but only partially inhibited killing of Renca. By contrast, the perforin inhibitor CMA significantly reduced NK cell lysis of Renca but not R331. Taken together, these data indicate that lysis of Renca by liver NK cells of poly(I:C)-treated mice predominantly occurs via the perforin lytic pathway. By contrast, R331 lysis was mediated by death ligands, and perforin-mediated lysis was minimal. Indeed, the immature TRAIL$^+$ NK cell population that is present in the liver but not in other organs (31) is probably mostly responsible for lysis of R331 cells.

To extend these findings, we also used liver effector NK cells from mice that had been injected with the cDNA for IL-2 by hydrodynamic injection. This procedure results in a dramatic increase in numbers of NK cells obtained from the liver. These NK cells may also have a higher level of activation based on an increased cell size and cell surface marker profile (28). Purified WT liver NK cells (>90% pure by phenotypic analysis) efficiently lysed Renca cells, and blocking Abs to FasL and TRAIL had little effect

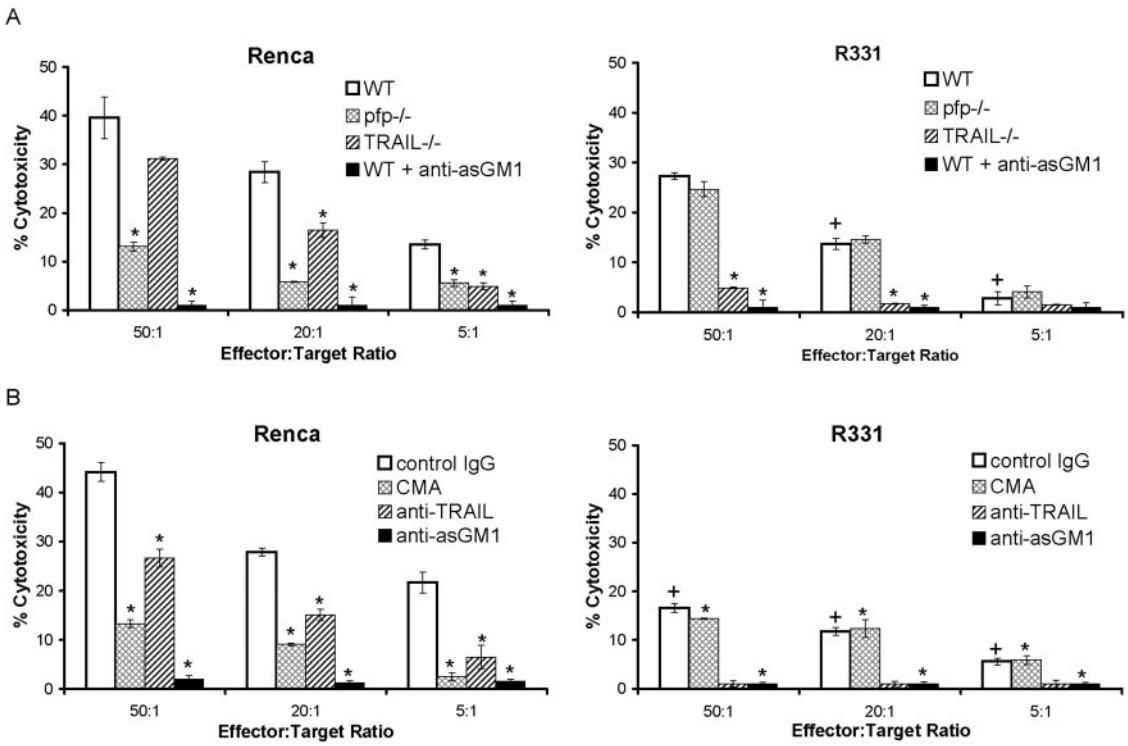

FIGURE 2. Lysis of Renca and R331 targets by liver NK cells. *A*, Liver mononuclear cells from poly(I:C)-treated BALB/c WT, pfp$^{-/-}$, TRAIL$^{-/-}$, or WT mice depleted of NK cells in vivo by anti-asGM1 treatment were used as effector cells. Data are recorded as cytolysis ± SD with significant differences between WT and other groups as defined by the Student's *t* test; ∗, $p < 0.005$. Differences between lysis of R331 and Renca; +, $p < 0.005$. *B*, BALB/c RAG1$^{-/-}$ liver NK cells in the presence of medium, anti-TRAIL, or following in vivo depletion of NK cell by anti-asGM1 treatment were used as effector cells in an 18-h cytotoxicity assay with Renca and R331 cells as targets. Data are recorded as cytolysis ± SD with significant differences between control IgG treatment and other groups as defined by the Student's *t* test; ∗, $p < 0.005$. Differences between lysis of R331 and Renca; +, $p < 0.005$.

(Fig. 3*A*). Lysis of R331 cells was much lower, yet could be augmented by addition of IL-2 and IL-18 to the cytotoxicity assay. However, this cytokine-mediated amplification of R331 lysis was blocked efficiently by anti-FasL. Liver NK cells from $pfp^{-/-}$ mice could only efficiently lyse either Renca or R331 following IL-2 and IL-18 treatment (Fig. 3*B*), and this could be blocked by Abs to FasL. It is interesting to note that activation of liver NK killing in response to IL-2 and IL-18 treatment seems to predominantly occur via the FasL pathway with a minor contribution from TRAIL. This result suggests that the NK cell population isolated from the livers of mice following treatment with cDNA for IL-2 has only low numbers of immature $TRAIL^+$ NK cells, but can use FasL as a cytolytic effector molecule when stimulated with IL-2 and IL-18. Concerning Renca cells, the use of $pfp^{-/-}$ NK cells seems to unmask the contribution of death ligands to Renca lysis. Therefore, death ligand-mediated lysis of Renca by NK cells only becomes readily apparent in the absence of perforin.

Role of NKG2D recognition of NKG2DL in the lysis of Renca and R331

One possible reason for the resistance of R331 cells to perforin-mediated cell death could be an intrinsic resistance of these cells to effector molecules such as perforin that are contained within the lytic granules. However, both Renca and R331 were equally susceptible to lysis by isolated granules (data not shown). Furthermore, NK cells cultured for extended periods in IL-2 develop a lymphokine-activated killer phenotype and can kill many targets by granule-mediated lysis. In contrast to the in vivo-derived NK cells used in this study, lymphokine-activated killer cells isolated from BALB/c *gld.gld* mice could kill both Renca and R331 cells efficiently in a perforin-dependent manner (data not shown). Therefore, the resistance of R331 cells to granule-mediated killing is relative and not absolute, and likely depends on the activation state of the NK cell effector population. Thus, both Renca and R331 cells can be sensitive to the lytic effects of NK granules, but the triggering of granule release does not occur efficiently on contact between in vivo-derived NK cells and R331. Because NKG2D seemed to be important for recognition of Renca cells in vivo, we assessed the effects of neutralizing Abs to NKG2D on lysis of Renca and R331 by NK cells. As seen in Fig. 4*A*, each of two neutralizing mAbs to NKG2D (A10 and C7) very efficiently blocked lysis of Renca by purified liver NK cells in a dose-dependent manner. As previously noted, lysis of R331 was very low in the absence of cytokine treatment. Inclusion of IL-2 and IL-18 in the cytotoxicity assay could activate NK cells to kill R331 targets in a death ligand-dependent manner (Fig. 3*A*), but this lysis was not affected by Abs to NKG2D (Fig. 4*B*). This suggests that the NKG2D recognition structure is important for triggering NK cell perforin-mediated killing, yet plays no role in death ligand-mediated lysis following IL-2 and IL-18 treatment.

Ligands involved in the recognition of Renca and R331 cells by NK cells

The preceding in vitro and in vivo data suggested that NKG2D-mediated recognition was crucial for signaling the perforin-mediated killing of Renca cells, yet this signaling did not appear to function well when R331 cells were the NK targets. We therefore attempted to examine the status of the known NKG2DL on Renca and R331 cells. RT-PCR using primers for H60, Rae1 family members, and MULT1 showed no obvious differences between Renca and R331 (data not shown). However, expression of mRNA may not necessarily correlate with the cell surface expression of protein. Interestingly, NKG2D tetramers bound to both Renca and

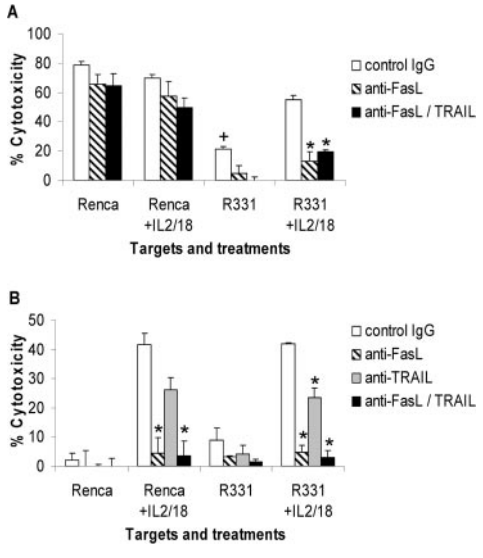

FIGURE 3. Lysis of Renca and R331 cells by liver NK cells from IL-2-treated mice. Purified liver NK cells from WT mice (*A*) or $pfp^{-/-}$ mice (*B*) isolated after injection of DNA for IL-2 were tested for their cytotoxic activity against Renca and R331 tumor cells in the presence or absence of 20 μg/ml neutralizing Abs to mouse FasL (MFL-1), TRAIL (N2B2), or a combination of both Abs at an E:T ratio of 25:1. Significant differences between control IgG and Ab treatments; $*$, $p < 0.005$. Significant differences between R331 and Renca; $+$, $p < 0.005$.

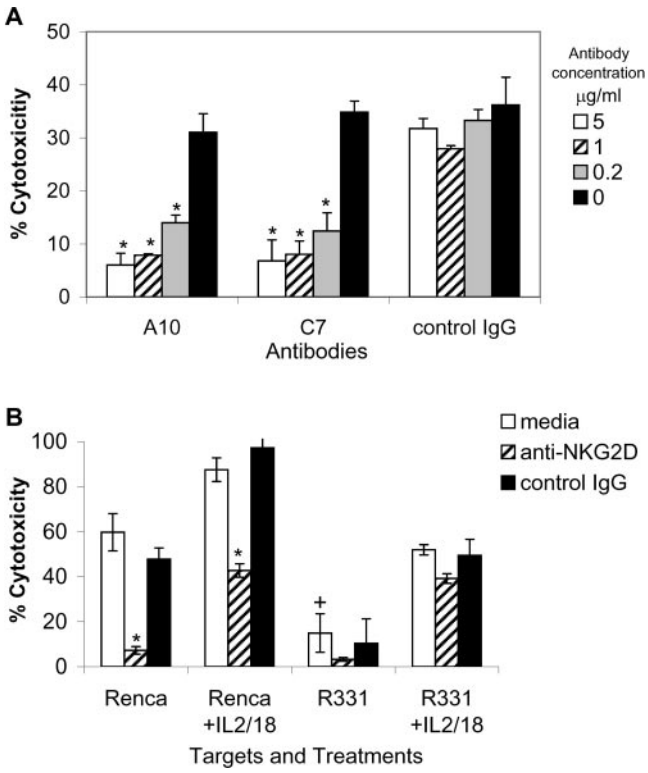

FIGURE 4. NK cell-mediated lysis of Renca in the presence of anti-NKG2D. Purified liver NK cells isolated from IL-2-treated mice were tested for their cytotoxic activity against Renca cells (*A*) in the presence or absence of various concentrations of the anti-NKG2D Abs A10 and C7. *B*, Renca and R331 cells in the presence or absence of IL-2 (500 U/ml) and IL-12 (10 ng/ml) and anti-NKG2D (C7) or control Ab at 20 μg/ml at an E:T ratio of 25:1. Significant differences between control IgG and test Abs; $*$, $p < 0.005$. Significant differences between R331 and Renca; $+$, $p < 0.005$.

R331 equally, suggesting that most of the ligands for NKG2D were present on both cell lines (Fig. 5). To investigate cell surface expression of NKG2DL, Renca and R331 were stained with mAbs to H60, a pan-Rae-1 Ab, and the CX1 mAb, and then examined by FACS analysis. No differences were observed on staining Renca or R331 with anti-H60 or pan-Rae-1 Abs. Interestingly, the CX1 Ab consistently exhibited a higher level of staining of Renca cells over R331 cells. Because this Ab is reported to bind most strongly with Rae1-γ, this suggests a somewhat higher level of expression of certain specific Rae-1 family members on Renca cells. Attempts to block lysis of Renca with the CX1 Ab were unsuccessful. In contrast to the minor differences observed for levels of known NKG2DL, there were more dramatic differences in the expression of ICAM-1 between Renca and R331 cells (Fig. 6). Renca constitutively expressed easily detectable cell surface ICAM-1 by FACS analysis, which could be further augmented on treatment with IFN-γ or a combination of IFN-γ and TNF-α. By contrast, constitutive expression of ICAM-1 on most R331 cells was negligible, and only a low percentage of cells expressed detectable ICAM-1 even following cytokine treatment. Therefore, the majority of R331 cells expressed little or no ICAM-1 on their cell surface and had lower levels of certain Rae-1 ligands. Because both of these defects may influence granule-mediated lysis by NK cells, we transfected R331 cells with Rae-1γ, ICAM-1, or Rae-1γ plus ICAM-1. Transfectants expressing high surface expression of either or both of these ligands were then isolated by cell sorting, and cell surface expression of transfected ligands was confirmed on FACS analysis (Fig. 7).

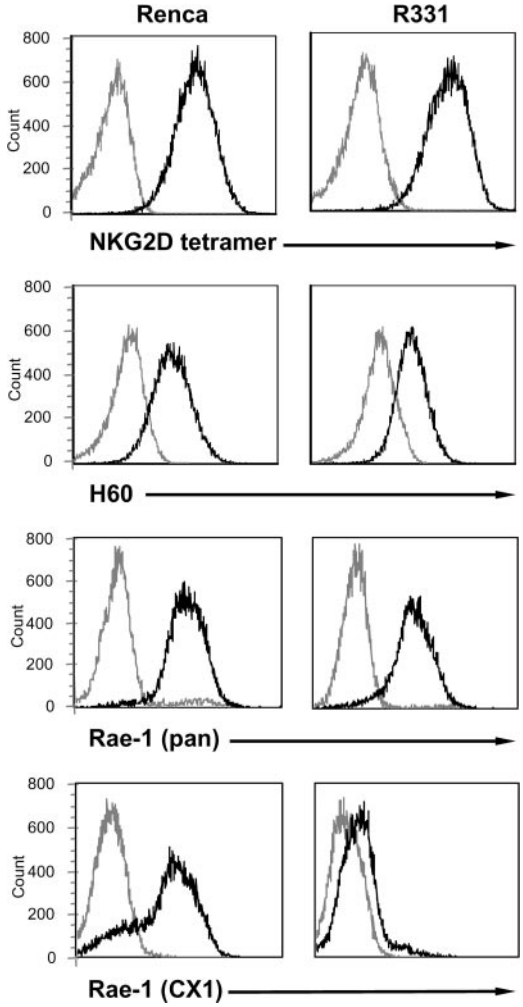

FIGURE 5. Differences in expression of NKG2DL on Renca and R331. Renca and R331 cells were stained with NKG2D tetramers, mAb to mouse H60, the pan-Rae1 mAb, or a more specific Rae1 mAb (CX1) followed by FACS analysis as described in *Materials and Methods*.

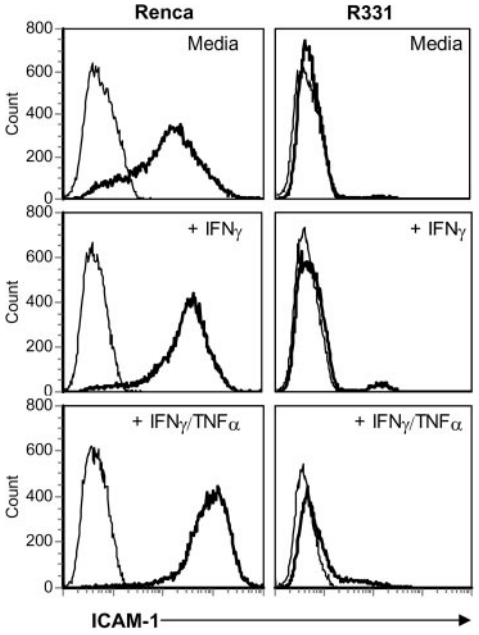

FIGURE 6. Differences in expression of ICAM-1 between Renca and R331. Renca and R331 cells untreated or treated overnight with IFN-γ (500 U/ml), TNF-α (500 U/ml), or a combination of both IFN-γ and TNF-α were stained with Abs to ICAM-1 followed by FACS analysis.

Lysis of R331-Rae1γ and -ICAM transfectants by NK cells

To determine whether ICAM-1 played any role in NK cell lysis of Renca, blocking Abs to CD18 (the common β_2 chain of the integrins LFA-1 and Mac-1) were included in the cytotoxicity assay. As seen in Fig. 8*A*, anti-CD18 could significantly block the lysis of Renca in 18-h cytotoxicity assays, when using effector cells from the livers of mice treated with cDNA-encoding IL-2, suggesting that NK cell integrins were important for NK cell lysis of Renca. Concerning the various transfectants, R331-Rae-1γ or R331-vector controls were only weakly lysed by liver NK cells from poly(I:C)-treated Rag-$2^{-/-}$ mice (Fig. 8*B*), at levels similar to R331 cells (data not shown). By contrast, the lysis of Renca, R331-ICAM and R331-ICAM-Rae-1γ transfectants was almost identical with lysis of Renca (Fig. 8*B*). Furthermore, this increase in lysis of both R331-ICAM and R331-ICAM-Rae-1γ transfectants could be almost totally blocked by anti-NKG2D or CMA (Fig. 8*C*). Therefore, increased expression of ICAM-1 rather than Rae-1γ on R331 cells was crucial for enhancing NK cytolysis, and this increased lysis was both perforin- and NKG2D-dependent. These patterns of target cell lysis were still maintained when liver NK cells from mice treated with cDNA for IL-2, or spleen NK cells from poly(I:C)-treated mice, were used as effectors (Fig. 8*C*). Furthermore, neutralizing Abs to FasL and TRAIL had no effect on lysis Renca or R331-ICAM-Rae-1γ (data not shown). Taken together, these data suggest that triggering of perforin-mediated killing of Renca cells in dependent on NKG2D-NKG2DL recognition. However, ICAM-1 expression by these target cells is necessary for optimal perforin-mediated lysis by NK cells directly isolated from mice.

Discussion

In this study, we have demonstrated distinct differences between Renca and R331 cells in triggering of perforin-mediated lysis by

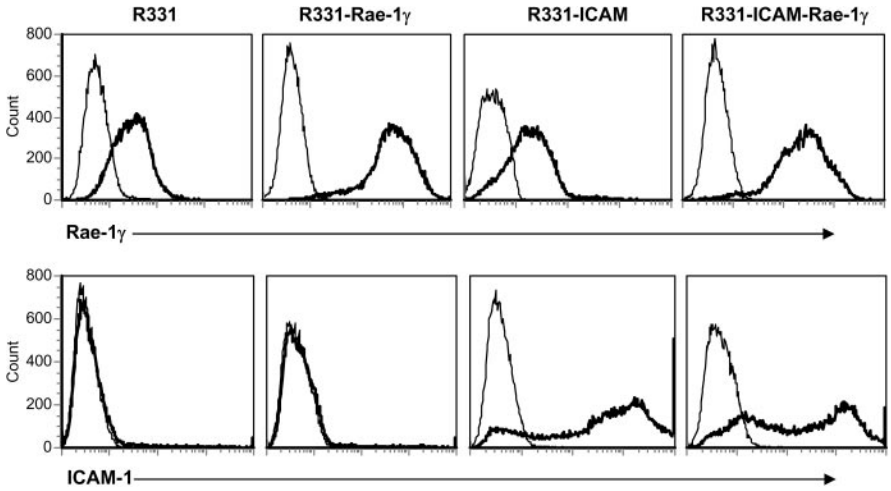

FIGURE 7. R331 transfectants expressing Rae-1γ, ICAM-1, and Rae-1γ plus ICAM-1. R331 transfectants for Rae-1γ, ICAM-1, or Rae-1γ plus ICAM-1, and their appropriate vector controls were sorted and analyzed by FACS after staining with appropriate Abs.

NK cells. These striking phenotypic differences have been used to further dissect the molecular requirements for NK cell cytotoxicity for both perforin and death ligand-mediated cytolysis. For perforin-mediated lysis of Renca, NKG2D-NKG2DL interaction is clearly crucial. However, R331 cells express multiple NKG2DL yet only trigger low levels of perforin-dependent cytotoxicity by NK cells, even when one of these ligands (Rae-1γ) is overexpressed. This suggests that while NKG2D-NKG2DL recognition by NK cells is necessary, this in and of itself is not sufficient to optimally trigger perforin-mediated lysis. Our studies on R331 cells, as well as the inhibition of Renca lysis by anti-CD18, suggest that an interaction between NK cell integrins and ICAM −1 is required to optimize perforin-mediated lysis.

Recently, an elegant series of studies have sought to determine the contribution of individual receptor-ligand interactions in NK cytotoxicity by overexpressing various ligands in insect target cells. Expression of ICAM-1 on the insect cells resulted in increased adhesion of human NK cells as expected (32), and a

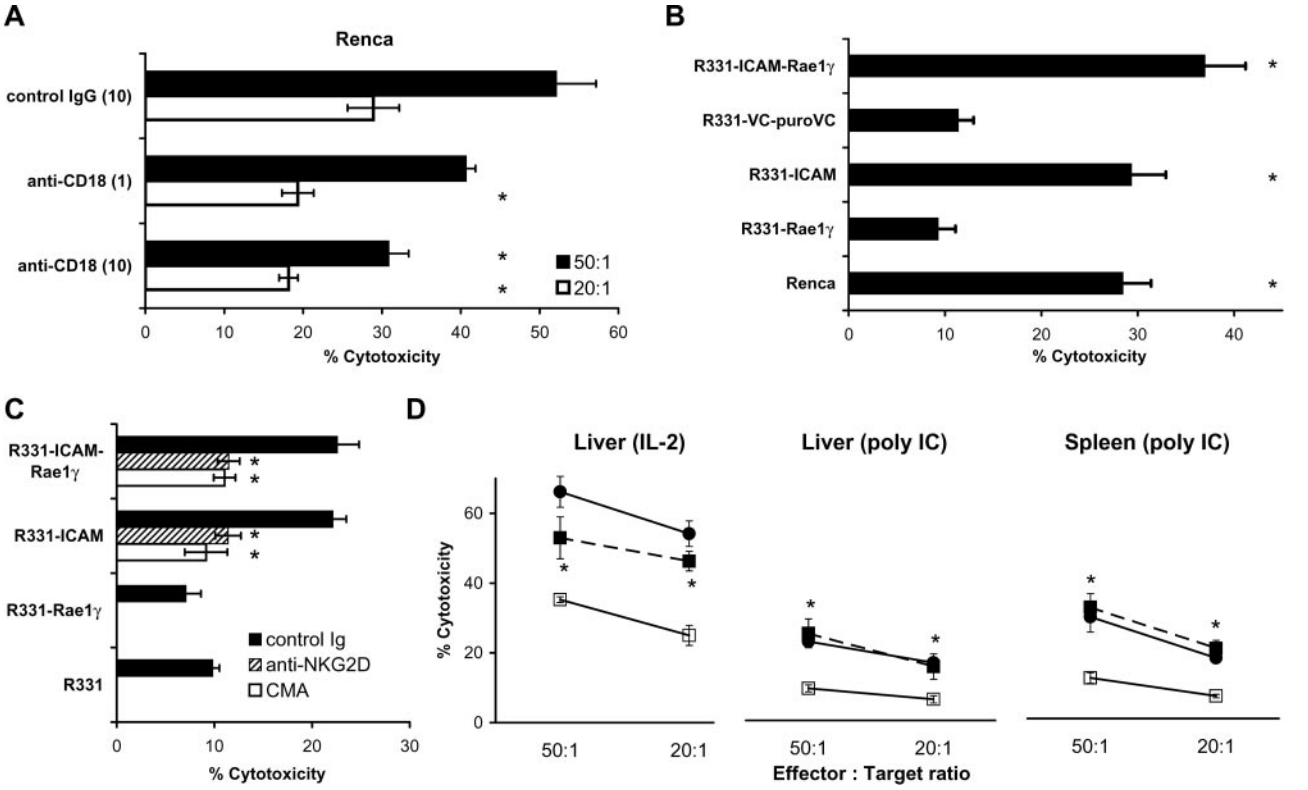

FIGURE 8. NK cell lysis of R331 transfectants. *A*, Liver mononuclear cells isolated from IL-2-treated mice were tested for their cytotoxicity against Renca target cells in the presence or absence of control IgG (10 μg/ml) or anti-CD18 (10 or 1 μg/ml). Significant differences between control IgG and anti-CD18; ∗, $p < 0.005$. *B*, Cytotoxic activity of liver mononuclear cells isolated from poly(I:C)-treated mice against Renca and various R331 transfectants (E:T ratio 40:1). Lysis significantly greater than R331 vector control; ∗, $p < 0.005$. *C*, Cytotoxic activity of spleen cells from poly(I:C)-treated mice against various R331 transfectants (E:T ratio 40:1) in the presence or absence of anti-NKG2D (20 μg/ml) or CMA (50 ng/ml). Lysis significantly inhibited by the treatments; ∗, $p < 0.005$. *D*, Cytolytic activity of liver mononuclear cells of IL-2-treated mice, and liver and spleen cells of poly(I:C)-treated mice against Renca (–○–), R331-ICAM-Rae1γ (–■–), or R331-VC-puroVC (–□–) targets. Significantly greater killing of all targets compared with R331-VC-puroVC; ∗, $p < 0.005$.

polarization of the NK lytic granules toward the ICAM-1-expressing target cells (33). Because this polarization could also be observed with beads coated with ICAM-1, ICAM-1 engagement alone was sufficient for granule polarization. Inhibitors of intracellular signaling could block the granule polarization without affecting the adhesion, indicating that binding to ICAM-1 triggered an intracellular signaling pathway in NK cells necessary for granule polarization (34). However, resting human NK cells required additional signaling following ICAM-1 binding for degranulation. Only when signals were present for both polarization (ICAM-1) and degranulation (CD16) did lysis of insect target cells occur (33). This data would be consistent with our findings. Therefore, both Renca and R331 recognition would be mediated by NKG2D/NKG2DL. However, this interaction would not be optimal for triggering granule polarization and subsequent lysis of R331 targets due to their low levels of ICAM-1, particularly when using resting NK cell effector populations. By contrast, more activated NK cells with higher lytic capacity have increased expression of the integrin Mac-1 that binds to ICAM-1 (35), and thus may still be able to respond to some degree to the very low levels of target cell ICAM-1 present on R331. Alternatively, signaling through NKG2D is known to be quite versatile. In the mouse, two splice variants of NKG2D are expressed (36). One isoform NKG2DL is only associated with signaling molecule DAP-10, whereas the other NKG2DS can associate with DAP10 and DAP12. Furthermore, levels of the NKG2DS isoform increases upon activation of NK cells with IL-2 (37). It is therefore tempting to speculate that signaling through NKG2D differs in activated NK cells due to an altered coupling to signaling molecules, and that this enhancement of signaling may thus override any requirements for other accessory ligands such as ICAM-1.

When cytokine-activated NK used the death ligand pathway to lyse Renca or R331 cells, there appeared to be no role for NKG2D-NKG2DL recognition. Our preliminary data indicated that the expression of FasL by the effectors cells was crucial, as was reported in earlier studies (38, 39). Furthermore, this suggests that the intracellular signaling pathways for death ligand and perforin-mediated cytolysis by NK cells are quite distinct. In this respect, our findings diverge somewhat from those of Bryceson et al., where cell surface FasL expression on human NK cells and degranulation occurred in response to the same signals. However, in their aforementioned study, cell surface increases in FasL on human NK cells occurred in response to ICAM-1 and Fc receptor (CD16) engagement. Using mouse NK cells, we did not see any dependence of FasL-mediated lysis on NKG2D and ICAM-1. Different signaling requirements for death ligand and granule-mediated lysis have been described for CTLs (40–42). Nonetheless, it has been reported by others (43) that expression of cell surface FasL and degranulation of T cells were both triggered by the same signals, and that FasL was stored in the lytic granules. However, the location of FasL in lytic granules has been questioned in subsequent studies (44). It is difficult to reconcile a lytic granule location for FasL with studies showing differing signaling requirements for death ligand and granule-mediated lysis. Our findings with mouse NK cells would be consistent with distinct signaling pathways controlling perforin and death ligand lytic effector functions.

A better understanding of all the structures involved in recognition of tumor cells by NK cells, and the subsequent triggering of lytic effector functions may allow for the design of improved immunotherapeutic strategies. For example, because Renca cells can be killed by both perforin and death-ligand pathways, a combination therapy with IL-2 and IL-18 may offer significant therapeutic benefits over either cytokine alone. Therefore, additional activation of death ligand-mediated cytolysis by NK cells may also facilitate the destruction of tumor variants (such as R331) that have evaded perforin-mediated cytolysis.

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Disclosures

The authors have no financial conflict of interest.

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