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Recognition and Prevention of Tumor Metastasis by the NK Receptor NKp46/NCR1

Ariella Glasner, Hormas Ghadially, Chamutal Gur, Noa Stanietsky, Pinchas Tsukerman, Jonatan Enk, and Ofer Mandelboim

NK cells employ a variety of activating receptors to kill virally infected and tumor cells. Prominent among these receptors are the natural cytotoxicity receptors (NCRs) (NKp30, NKp44, and NKp46), of which only NKp46 has a mouse ortholog (NCR1). The tumor ligand(s) of NKp46/NCR1 is still unknown, but it was shown that the human NKp46 and the mouse NCR1 are involved in tumor eradication both in vitro and in vivo. Whether any of the NK activating receptors is involved in the prevention of tumor metastasis is unknown. To address this question, we studied the activity of the NK cell receptor NKp46/NCR1 in two spontaneous metastasis models, the B16F10.9 melanoma (B16) and the Lewis lung carcinoma (D122) in the NCR1 knockout mouse that was generated by our group, in various in vitro and in vivo assays. We demonstrated that all B16 and D122 tumors, including those generated in vivo, express an unknown ligand(s) for NKp46/NCR1. We have characterized the properties of the NKp46/NCR1 ligand(s) and demonstrated that NKp46/NCR1 is directly involved in the killing of B16 and D122 cells. Importantly, we showed in vivo that NKp46/NCR1 plays an important role in controlling B16 and D122 metastasis. Thus, to our knowledge, in this study we provide the first evidence for the direct involvement of a specific NK killer receptor in preventing tumor metastasis. ***The Journal of Immunology*****, 2012, 188: 2509–2515.**

Natural killer cells make up to 15% of all PBLs. They kill cells that either express insufficient amounts of MHC class I ("missing self" hypothesis) (1) or extensively express NK killer ligands. They are cytotoxic to virus-infected cells and to a wide range of tumors (2), and their activity is regulated through a balance of signals derived from inhibitory and activating receptors (3). The activating NK cell receptors include proteins such as CD16, NKp80, 2B4, NKG2D, and the natural cytotoxicity receptors (NCRs) NKp46, NKp44, and NKp30 (4, 5). Although it has been recently demonstrated that NKp46 is expressed on a special subset of lymphocytes of the innate immune system (6), NKp46 is still considered a unique NK cell marker and is the only NCR that has a mouse ortholog, named NCR1 (4, 7). Interestingly, although the influenza virus hemagglutinin (HA) was identified as a ligand of NKp46/NCR1 (7, 8), the identity of the tumor ligand(s) of this important receptor is still elusive.

Tumorigenesis is a multistep processes in which cancer cells, which are generated due to environmental, genetic, infection, or chronic inflammatory conditions, undergo various changes (some of which are influenced by the immune system), which in most cases result in tumor metastasis (9, 10). Most cancer patients often succumb to metastasis, which is the major cause of cancer-related lethality (11). NKp46/NCR1 was shown to take an important part in controlling the spread of various primary tumors, including melanoma, lymphoma, carcinoma, and carcinogen-induced fibrosarcoma (3-methylcholanthrene [MCA]-induced) (12–14). However, it is still unknown whether any of the NK activating receptors is directly involved in the regulation of spontaneous tumor metastasis. In this study, to our knowledge we provide the first evidence for the direct involvement of a specific NK killer receptor in preventing spontaneous tumor metastasis.

Materials and Methods

Mice, tumor development, and metastasis

All experiments were performed using 6- to 8-wk-old mice of the C57BL/6 background. The generation of the NCR1 knockout (KO) mice NCR1$^{gfp/gfp}$ was described previously (7). All experiments were performed in a specific pathogen-free unit of the Hebrew University Medical School (Ein-Kerem, Jerusalem, Israel) in accordance with the guidelines of the Ethics Committee. Mice were injected with 1×10^5 B16F10.9 (B16) or Lewis lung carcinoma (D122) cells into the s.c. space of the right footpad. Developing tumors were visually inspected daily. Once a tumor reached a volume of 100 mm^3 it was surgically removed. All survival experiments were performed double-blind, and all mice were monitored daily. No differences were observed between the wild-type (WT) and KO mice in signs of fever, infection, or in their general health, until the point of dying. Each mouse that died was autopsied and the existence of metastases was visually verified. Furthermore, no mouse that survived showed the abovementioned symptoms at any time point. The mice that survived were examined for metastases after the termination of the experiments (>120 d following the primary tumor excision), and all these mice were metastases free. In the experimental metastasis assay, 1×10^5 or fewer D122 cells were injected into the tail vein. Mice were monitored thereafter daily, and survival was assessed. Macrometastases were counted by the researchers, as the metastatic foci are very well defined in shape, color, and general appearance.

Cells

Murine B16F10.9 (B16) and Lewis lung carcinoma (D122) cells were provided by Prof. Lea Eisenbach (The Weizmann Institute of Science,

Lautenberg Center for General and Tumor Immunology, Institute for Medical Research Israel–Canada, Hebrew University–Hadassah Medical School, 91120 Jerusalem, Israel

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Address correspondence and reprint requests to Prof. Ofer Mandelboim, Lautenberg Center for General and Tumor Immunology, Institute for Medical Research Israel–Canada, Hadassah Medical School, P.O. Box 12272, 91120 Jerusalem, Israel. E-mail address: oferm@ekmd.huji.ac.il

Abbreviations used in this article: HA, hemagglutinin; KO, knockout; MCA, 3-methylcholanthrene; NA, neuraminidase; NCR, natural cytotoxicity receptor; PK, proteinase K; poly(I:C), polyinosinic-polycytidylic acid; TC, tissue culture; WT, wild-type.

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Rehovot, Israel) and Prof. Shamgar Ben-Elyahu (Tel Aviv University, Tel Aviv, Israel). Other cell lines used in this study were the murine thymoma BW, BW-NCR1-ζ, and BW-CD16-ζ (8), the murine virus-induced thymic lymphoma PD1.6, the human cervical adenocarcinoma HeLa, the murine lymphoma YAC-1, and the EBV-transformed B cell lymphoma 721.221. To obtain cell lines from the primary tumors or from lung metastases, samples of the tissues were treated with 3 ml trypsin for 1 min, washed, and placed in culture. Two days later tissues were removed and cells were grown in complete DMEM.

Fusion proteins, Abs, and flow cytometry

The NCR1-Ig and the NKp46-D1-Ig fusion proteins were generated in COS-7 cells, as previously described. Anti-HA1 H17-L2 was a gift from Jonathan W. Yewdell (National Institutes of Health, Bethesda, MD). Treatment of the fusion proteins with neuraminidase (NA) was performed as described (15). For the proteinase K (PK) treatment, cells were incubated for 20 min with 10 μl PK (20 mg/ml; Sigma-Aldrich, Rehovot, Israel). The staining of all cell lines by fusion proteins was visualized using a secondary PE- or allophycocyanin-conjugated goat anti-human Ab (Jackson ImmunoResearch Laboratories).

CD107a, killing, and cytokine secretion assays

Mice were injected i.p. with 200 μg polyinosinic-polycytidylic acid (poly(I:C); Sigma-Aldrich) and PBLs were harvested 18 h later. For the CD107a assay, 5×10^5 PBLs were coincubated with the indicated target cells in a ratio of 1:1 in the presence of 0.1 μg allophycocyanin-conjugated CD107a Ab (Biotest, Kfar Saba, Israel) for 2 h, and CD107a levels on the NK cells were determined by flow cytometry. For the NK cytotoxicity assays, PBLs were harvested from WT and KO mice 18 h following poly(I:C) injection and incubated for 5 h with [^{35}S]methionine-labeled target cells at various E:T ratios. Cytotoxicity was quantified as previously described (8). For cytokine secretion, the activated PBLs were coincubated with radiated target cells (3000 rad) at a 1:1 ratio for 48 or 72 h at 37°C and 5% CO_2. Supernatants were collected 48 or 72 h later and IFN-γ or TNF-α levels were determined using IFN-γ and TNF-α Abs (BioLegend) and standard ELISA.

Statistical analyses

ANOVA was used to identify significant group differences. Provided group differences were indicated, and Fisher protected least significant differences contrasts were used to test specific pairwise comparisons with respect to an a priori hypothesis. To assess survival rates, the Kaplan–Meier model was used followed by the Tarone–Ware test for pairwise group comparisons. A p value of <0.05 was considered significant in all studies, and all p values were two-tailed.

Results

B16 and D122 cells express a ligand or ligands for NKp46/NCR1

To test whether NKp46/NCR1 plays a role in the control of spontaneous metastasis, we used the B16-derived cell line B16F10.9 (B16) and the Lewis lung carcinoma-derived cell line D122, which spontaneously metastasize to the lungs following the removal of a primary tumor, which had previously been injected into the footpad. Because the cellular ligand(s) for NKp46/NCR1 is yet unknown, we stained the B16 and D122 cells with fusion proteins consisting of the extracellular part of the NCR1 (the mouse ortholog of NKp46) fused to the FC part of human IgG1 (NCR1-Ig). As a control we used a fusion protein consisting of the membrane distal domain of the human NKp46 receptor fused to Ig (NKp46-D1-Ig), which was shown not to interact with the unknown human NKp46 ligand(s) (15). Because marked expression of the unknown NKp46/NCR1 ligand(s) was detected in B16 and D122 cells (Fig. 1A), we concluded that B16 and D122 cells express an unknown tumor ligand(s) for NKp46/NCR1.

To test whether the engagement of NKp46/NCR1 by its unknown ligand(s) expressed by B16 and D122 cells would lead to activation of the receptor, we used a cell-based reporter assay of BW cells expressing NCR1 fused to the mouse ζ-chain (BW-NCR1-ζ) (8). In this system, triggering of NCR1-ζ leads to IL-2 secretion, thus reporting for the functional interaction of NCR1 with its ligand(s). As controls we used BW and BW-CD16-ζ (prepared in a similar manner as NCR1-ζ) (8), and the expression of all receptors on the BW cells was verified by FACS (Fig. 1B). BW, BW CD16-ζ, and BW-NCR1-ζ cells were incubated with B16 and D122 cells, as well as with PD1.6 (a tumor known to express the NKp46/NCR1 ligand(s)) (13). As shown in Fig. 1C, a significant increase in the secretion of IL-2 was observed when the BW-NCR1-ζ cells were incubated with PD1.6, B16, and D122 cells, indicating that all three cell lines express a functional ligand(s) for NKp46/NCR1 ($p = 0.003$, $p = 0.04$, and $p = 0.012$, respectively).

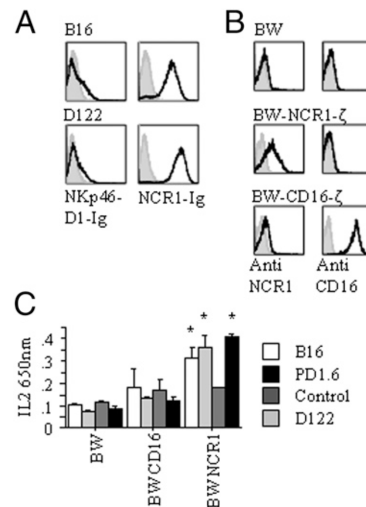

FIGURE 1. B16 and D122 cells express ligand(s) for NKp46/NCR1. (**A**) FACS staining of TC D122 cells performed either with NCR1-Ig (*right*) or with a control fusion protein NKp46-D1-Ig (*left*). The filled gray histogram is the background staining with a secondary Ab. Data from one representative of three independent experiments are shown. (**B**) FACS analysis of various BW and BW transfectants expressing either the chimeric NCR1-ζ receptor or the CD16-ζ protein using NCR1 and CD16 Abs, respectively. The filled gray histogram is the background staining with a secondary Ab. Data from one representative of two independent experiments are shown. (**C**) IL-2 secretion from BW, BW-CD16-ζ, or BW-NCR1-ζ cells following 48 h incubation with the indicated tumor cell line. Values are shown as means ± SEM. Three independent experiments were performed. Statistical analysis was calculated on the data from all experiments combined. *Statistically significant: PD1.6, $p = 0.003$; B16, $p = 0.04$; D122, $p = 0.012$.

NKp46/NCR1-dependent NK degranulation following incubation with B16 and D122

Activation of NKp46/NCR1 by its unknown tumor ligand(s) can lead either to IFN-γ secretion (14), to enhanced killing, or to both (7, 8, 12). To test which of these mechanisms is activated when NK cells interact with B16 or D122 cells we used the $NCR1^{gfp/gfp}$ (KO) and the immune competent $NCR1^{+/gfp}$ (Het) mice, (7), in a CD107a (LAMP-1) mobilization assay (16). Het and KO mice were injected i.p. with poly(I:C) and 18 h later, activated PBLs were incubated with B16, D122, PD1.6, YAC-1 (killed independently of NKp46/NCR1) (7, 13), and HeLa cells (as a negative control). We used Het mice in these experiments, as similarly to the KO mice, all NK cells in these mice express GFP and can easily be detected by FACS (7). As shown in Fig. 2A, NK cells from Het mice degranulated significantly more than did NK cells from KO mice after incubation with PD1.6, B16, and D122 cells ($p = 0.008$, $p = 0.005$, and $p = 0.001$, respectively). Such differences were not observed in the incubation

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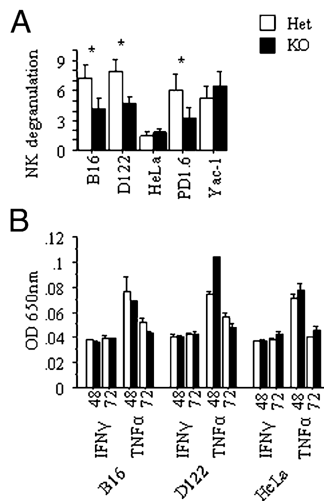

FIGURE 2. NKp46/NCR1-mediated degranulation. (**A**) Whole-blood PBLs were harvested 18 h following poly(I:C) injection into NCR1$^{+/gfp}$ (Het) and NCR1$^{gfp/gfp}$ KO mice and were incubated for 2 h with various tumor cell lines (as indicated, *x*-axis) together with a CD107a Ab. CD107a levels on GFP-marked NK cells were quantified by FACS, and the amount of CD107a expression on the Het NK cells that were incubated with no target was set as baseline. Values are shown as means ± SEM. Three independent experiments were performed. Statistical analysis was calculated on the data from all experiments combined. *Statistically significant: PD1.6, $p = 0.008$; B16, $p = 0.005$; D122, $p = 0.001$. (**B**) PBLs derived from Het and KO mice were incubated with irradiated (3000 rad) B16, D122, and HeLa target cells for 48 or 72 h. The presence of IFN-γ and TNF-α in the culture supernatants was measured by ELISA. Cytokine secretion level from Het NK cells incubated with no target was set as baseline. Values are shown as means ± SEM. Three independent experiments were performed. Statistical analysis was calculated on the data from all experiments combined.

with YAC-1 or HeLa cells (Fig. 2A). We also tested whether the interactions between NK cells and B16 or D122 cells would lead to IFN-γ or TNF-α secretion, and we observed no effect (Fig. 2B). Thus, we concluded that the interaction between the NKp46/NCR1 and its unknown ligand(s) expressed by B16 and D122 cells leads to NK cell degranulation, but not to cytokine production by NK cells.

NKp46/NCR1 is involved in the control of B16 and D122 metastasis

To test whether NKp46/NCR1 plays a role in controlling tumor growth in vivo, we injected 1×10^5 B16 or D122 cells into the footpad of NCR1 KO and WT littermates. Once a tumor reached a 100 mm^3 volume it was excised (Fig. 3A). No differences were found between the WT and KO mice in signs of fever, infection, or in general health following the injection of the tumor cells, and we detected no significant difference in tumor occurrence or growth rate of the B16 or D122 tumors between WT and KO mice (Fig. 3A). We next wondered whether metastases formation would be influenced by the absence of NCR1, and to test this we initially weighed the lungs 28 d following the removal of the primary tumors. When the weight of the lungs was compared, we noticed that the KO mice had significantly heavier lungs than did the WT mice in both models (Fig. 3B: B16, $p = 0.003$; D122, $p = 0.007$). We therefore speculated that the KO lungs might be heavier due to the existence of micrometastases. Still, however, the difference in lung weight at day 28 could be attributed to various reasons other than the onset of the metastatic process, such as increased inflammation in the KO mice due to the inability of their NK cells to control the injected tumor cells. Thus, to elucidate the role of NKp46/NCR1 in controlling metastasis, we performed survival experiments in which mortality rates were monitored following the excision of the primary tumors.

Importantly, in these survival experiments no differences were observed between the WT and KO mice in signs of fever, infection, or in general health following the injection of the tumor cells or after the removal of the primary tumors, until the point of dying. Each mouse was autopsied postmortem and the existence of metastases was visually verified. Notably, in these survival assays, KO mice had significantly worse overall survival rates compared with the WT mice in both models (Fig. 3C: B16, $p = 0.04$; D122, $p = 0.01$). Furthermore, lung metastases were counted and, as shown in Fig. 3D, the increased incidence of death in the KO mice was associated with increased metastatic foci (most KO animals that died had >20 metastases), and only negligible percentages had fewer metastases, regardless of genotype (Fig. 3D).

The survival rate of KO mice was significantly worse in both models (Fig. 3C); however, in the D122 model the effect was markedly larger. For that reason, and due to ethical considerations (i.e., to minimize the sacrifice of test animals), from this point on we focused on the D122 model only.

Immune editing and tumor ligand(s) characteristics

The results presented so far were quite surprising, as we had shown that NKp46/NCR1 is involved in preventing spontaneous metastasis, but not in the control of the primary tumors. Therefore in the next sets of experiments we tried to elucidate the mechanism(s) accounting for these differences.

One possible explanation for the involvement of the NKp46/NCR1 in metastases eradication, but not in the primary tumor development, might be that the ligand has changed in vivo during metastasis. To investigate this we prepared cell lines from D122 primary tumors or metastases that were grown in either WT or KO mice, and stained them with the NCR1-Ig fusion proteins (Fig. 4). Alhough some of these lines showed reduced expression of the unknown NKp46/NCR1 ligand(s) (Fig. 4A, 24-9; Fig. 4C, 76; and Fig. 4D, 75n), this reduction in expression was not consistent and probably occurred due to tumor heterogeneity.

Our next hypothesis was that qualitative differences between the D122 primary tumors and metastases were responsible for the differences observed in NKp46/NCR1 dependencies, and we therefore investigated the biochemical/molecular properties of the unknown NKp46/NCR1 ligand(s). It was previously shown that both the human NKp46 and the mouse NCR1 recognize the influenza virus HA in a sialic acid-dependent manner (7, 8, 15, 17). To study whether the unknown NKp46/NCR1 ligand(s) expressed on D122 cells is a lectin, with properties similar to those of HA, we treated the NCR1-Ig fusion proteins with NA to remove sialic acid residues. Such treatment, as reported (15), led to the abrogation of the binding of NCR1-Ig to PR8 influenza-infected 721.221 cells (Fig. 5A, *upper panel*). Influenza infection of 721.221 cells was verified by staining with anti-HA Ab (Fig. 5A, *lower panel*). However, NA treatment did not affect the binding of NCR1-Ig to tissue culture (TC) D122 or PD1.6 cells (Fig. 5B), indicating that the unknown NKp46/NCR1 ligand(s) expressed by D122 and PD1.6 cells is not a sialic acid-binding lectin. In contrast, treatment of the TC D122 and PD1.6 cells with PK abrogated the NCR1-Ig binding (Fig. 5B), indicating that the NKp46/NCR1 ligand(s) on these cells is a protein or a modification expressed on a protein.

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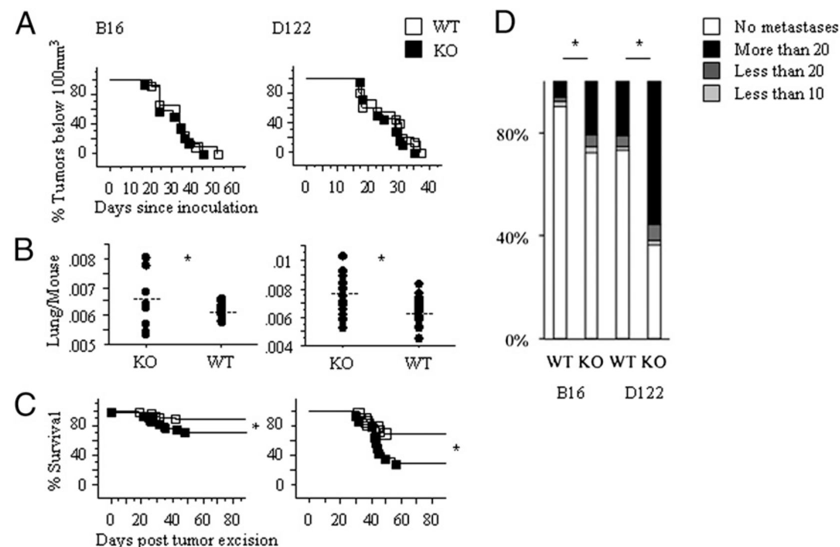

FIGURE 3. NKp46/NCR1 is involved in the control of B16 and D122 metastasis. (**A**) B16 or D122 tumor cells (1×10^5) were injected into the footpad of NCR1$^{+/+}$ WT and NCR1$^{gfp/gfp}$ C57BL/6 (KO) mice and tumor volume was monitored daily until tumors reached 100 mm^3, at which point tumors were surgically excised. At least 15 mice were used in each group, in three independent experiments. (**B**) WT and KO mice injected with the tumor cells were killed 28 d following the removal of the primary tumors, and lungs were harvested and weighed. The figure shows the ratio of lung/mouse weight. Horizontal lines indicate means. At least 15 mice were used in each group, in each of three independent experiments. Statistical analysis was calculated on the data from all experiments combined. *Statistically significant: B16, $p = 0.003$; D122, $p = 0.007$. (**C**) Survival assay. WT or KO mice that underwent tumor excision were monitored daily thereafter and survival was assessed using the Kaplan–Meier model. The termination point of the experiment was set to 120 d. At least 15 mice were used in each group, in each of three independent experiments. Statistical analysis was calculated on the data from all experiments combined. (**D**) Quantification of the number of lung metastases in mice that underwent the experiments presented in (C), at the time of death. Each mouse that died was autopsied and lung metastases were counted. *Statistically significant: B16, $p = 0.04$; D122, $p = 0.01$.

We then stained primary and metastatic D122 cells obtained from WT and KO mice with an NCR1-Ig fusion protein that was either treated with NA or not, and observed no significant differences (Fig. 5C). In contrast, treatment of the D122 tumors with PK resulted in a complete disappearance of the NKp46/NCR1 ligand(s) from all D122 tumors (Fig. 5C). This NA-resistant, PK-sensitive phenotype of the NKp46/NCR1 ligand(s) was observed in primary tumors and metastases that originated from the same animals (namely, WT mouse no. 38 or KO mouse no. 81) (Fig. 5C), indicating that in this regard, primary tumors and metastases do not differ.

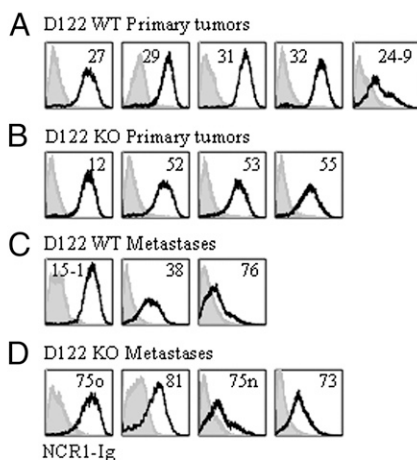

FIGURE 4. NKp46/NCR1 plays no significant role in the editing of D122 tumors. (**A**–**D**) Binding of NCR1-Ig to D122 primary tumors (A, B) or to D122 metastases (C, D) of WT (A, C) and KO (B, D) mice. The numbers indicate different animals. The gray filled histogram is the background staining with the control NKp46-D1-Ig. The dark line histograms represent specific staining with the NCR1-Ig fusion protein. Data from one representative of three independent stainings are shown.

NKp46/NCR1-dependent killing of primary and metastatic D122 tumors

Next, we asked whether the observed in vivo NKp46/NCR1 dependency of D122 metastases (Fig. 3C) would result in differences in killing in vitro. To investigate this, we used the CD107a degranulation assay with NK cells derived from Het or KO mice and various primary and metastases D122 tumors that originated either in the WT or in the KO mice. As shown in Fig. 6A, all D122 cells induced less CD107a mobilization in NK cells derived from the KO mice, irrespective of their origin (primary or metastatic tumors) and whether they had developed in the presence (WT) or absence (KO) of NKp46/NCR1 ($p < 0.001$ for all comparisons) (Fig. 6A).

CD107a degranulation was shown to correlate with NK and T cell killing (16); however, to directly demonstrate the ability of the NK cells to kill the various tumor cells in a NKp46/NCR1-dependent manner, we also performed a direct cytotoxicity assay using [^{35}S]methionine-labeled target cells and NK cells derived from the WT and KO mice. Labeled target cells derived from primary and metastatic D122 tumors were incubated with WT and KO NK cells and, as shown in Fig. 6B, all D122 target cells were better killed by NK cells derived from the WT mice, irrespective of their origin (primary or metastatic tumors) and whether they had developed in the presence (WT) or absence (KO) of NKp46/NCR1 ($p < 0.05$ for all comparisons). Additionally, NK cells derived from the WT mice killed the B16 cells better than did NK cells derived from the KO mice, although the absence of NCR1 had only a small effect on the B16 metastasis relative to the D122 metastasis (Fig 3C).

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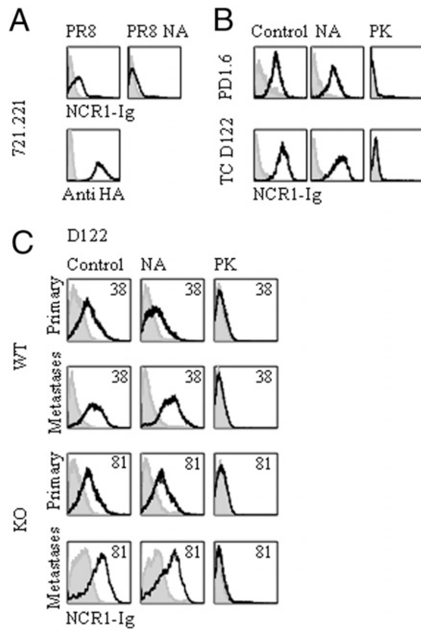

FIGURE 5. Characteristics of the NKp46/NCR1 ligand. (**A**) 721.221 cells were infected with influenza PR8 or left uninfected and then stained with NCR1-Ig (PR8, *upper*), with NCR1-Ig that was treated with NA (PR8 NA, *upper*), or with anti-HA1 (*lower*). The gray filled histogram is the background staining with a secondary Ab only (*bottom*) or with the control NKp46-D1-Ig (*upper*). The dark line histogram represents either the NCR1-Ig fusion protein staining (*upper*) or the staining with the anti-HA (*lower*). Data from one representative of three independent stainings are shown. (**B**) TC D122 (*lower*) or PD1.6 (*upper*) cells were stained with NCR1-Ig following treatment of the fusion proteins with NA or with PK. The gray filled histogram is the background staining with the control NKp46-D1-Ig. The dark line histogram represents the NCR1-Ig staining following the various treatments (NA or PK). Control is the staining of the cells with untreated NCR1-Ig. (**C**) Primary and metastatic D122 tumors growing in WT (*upper two rows*) or KO mice (*lower two rows*) were stained either with the untreated NCR1-Ig (control) or with NCR1-Ig treated with NA or with PK. Gray shading represents background staining of the control NKp46-D1-Ig; the solid line represents NCR1-Ig fusion protein staining. The numbers indicate the various mice. Data from one representative of three independent stainings are shown.

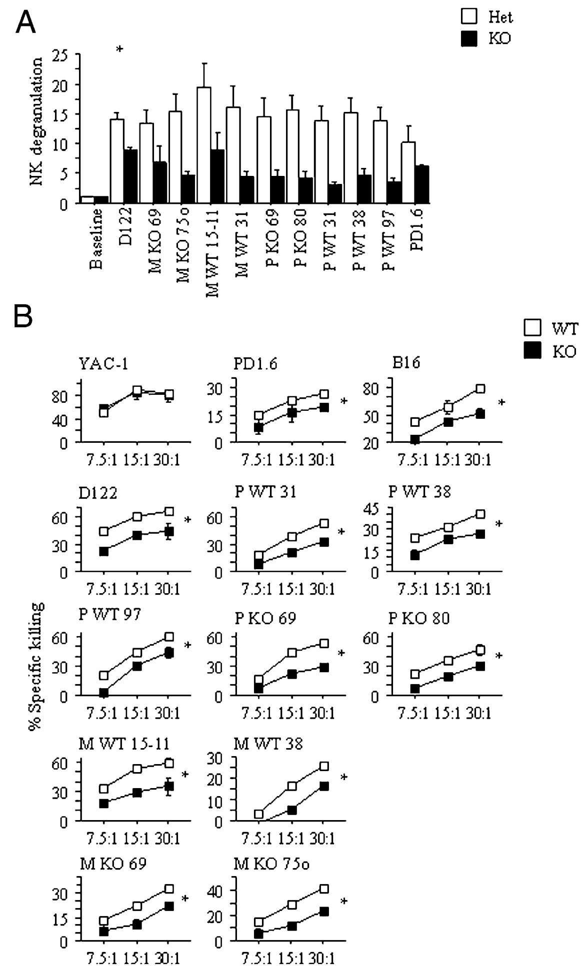

FIGURE 6. NK cell degranulation and killing of various D122 tumors. (**A**) Whole-blood PBLs were isolated 18 h following poly(I:C) injection into NCR1$^{gfp/-}$ (Het) and NCR1$^{gfp/gfp}$ (KO) mice. The PBLs were incubated with HeLa, PD1.6, and with the various D122 tumor cell lines of primary tumors (P) or metastases (M) derived either from NCR1$^{+/+}$ (WT) or from NCR1$^{gfp/gfp}$ (KO) mice (indicated at the *x*-axis), and CD107a levels were analyzed on the GFP-positive NK cells by FACS. Values are shown as means ± SEM. Three independent experiments were performed. Statistical analysis was calculated on the data from all experiments combined. *Statistically significant: $p < 0.001$ for all comparisons. (**B**) Whole-blood PBLs were isolated from NCR1$^{+/+}$ (WT) and NCR1$^{gfp/gfp}$ (KO) mice 18 h following poly(I:C) injection. The PBLs were incubated with labeled B16, YAC-1, PD1.6, and with the various D122 tumor cell lines of primary tumors (P) or metastatic (M) origin that were derived either from NCR1$^{+/+}$ (WT) or from NCR1$^{gfp/gfp}$ (KO) mice. Values are shown as means ± SEM. *Statistically significant: repeated-measures ANOVA indicated $p < 0.05$ for all comparisons.

The site of tumor inoculation affects the NKp46/NCR1 dependency

Because no differences were observed in D122 cells derived either from primary or metastatic tumors, irrespective of whether they had grown in the presence or absence of NKp46/NCR1, we wondered whether it is the tumor location that determines the NKp46/NCR1 dependency of the D122 metastases. Thus, we hypothesized that the NKp46/NCR1 receptor might be able to control the relatively small amount of micrometastases that enter the circulation following tumor excision; however, when the NKp46/NCR1 receptor encounters the initial mass of s.c. administered cells, it is overpowered by the larger number of tumor cells and is unable to contain them. To test this, we administered 1×10^5 (the same number of D122 cells that was originally injected intrafootpad) or less D122 cells i.v. into WT and KO mice. Notably, in this setting of "experimental" D122 metastasis, tumors failed to generate in either of the animals when $<1 \times 10^5$ D122 cells were injected (100% survival, data not shown). As shown in Fig. 7, when we injected 1×10^5 cells i.v., there was no difference in mortality between the WT and KO mice. The result is strikingly contradictory to the marked and significant difference we detected in the spontaneous metastasis setting in the experiments shown in Fig. 3B and 3C.

Discussion

The foremost cause of death in cancer patients is not by the primary tumors, as they are usually surgically removed, but rather by the development of metastases and the recurrence of the disease (18, 19). Interestingly, in many cancers, metastases are more likely to form only after the primary tumor is removed. This is caused by increased dissemination of tumor cells during the removal of the primary tumor (20), decreased levels of anti-angiogenic factors, local and systemic increases in proangiogenic and growth factors (e.g., VEGF) (21), blood loss (22), and the suppression of cell-

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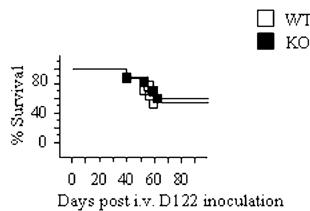

FIGURE 7. Experimental lung metastasis. NCR1$^{+/+}$ (WT) or NCR1$^{gfp/gfp}$ (KO) mice were i.v. administered 1×10^5 D122 cells and survival was monitored. Survival rates were compared using the Kaplan–Meier model. The termination point of the experiment was set to 120 d. At least 15 mice were used in each group. Two independent experiments were performed. Statistical analysis was calculated on the data from all experiments combined.

mediated immunity (following the surgery) (23, 24). Also, the primary tumor is known to secrete immunosuppressive factors such as PGE_2, IL-10 and TGF-β, which increase the likelihood of tumor cells to stay close to the initial mass of the primary tumor. Once the primary tumor is removed, tumor cells are no longer protected by its immunosuppressive environment and are more likely to migrate.

Peripheral metastases are usually numerous, more aggressive, often inoperable, and are less reactive to therapy, having undergone immune editing and escaping immune detection (25). Therefore, it is critical to understand whether and how cells of the immune system can control metastasis. In this study, to our knowledge we provide the first example of recognition and elimination of tumor metastases mediated by a specific NK activating receptor.

NK cells are effective against many pathogens and are predominantly distinguished by their ability to instantly kill transformed cells (26). Among the NK cell killer receptors, the NCRs NKp30, NKp44, and NKp46/NCR1, which were discovered more than a decade ago (4), were shown to be paramount to many immunological processes. Specifically, NKp46/NCR1 was implicated in influenza (7, 8), tumorigenesis (13), and diabetes (27, 28). However, remarkably, other than the influenza virus HA (7) and the pox virus HA (29), no ligands for this important receptor were identified. In this study, we show that NKp46/NCR1 recognizes an unknown ligand(s) expressed by the metastatic cell lines B16 and D122, as well as by D122 tumors and metastases. We demonstrate that unlike the HA protein that interacts with the sialic residues of the NKp46/NCR1 (7, 8, 17), the unknown D122 ligand(s) of NKp46/NCR1 binds this receptor in a sialic acid-independent manner. We further demonstrate that the tumor ligand(s) expressed by PD1.6 and D122 cells contains a protein component.

Given the diverse functions of the NKp46/NCR1, we think that the ligands for the NKp46/NCR1 are probably numerous and that the engagement of each would have different consequences. Indeed, whereas the influenza HA is a lectin, and its interaction with NKp46/NCR1 causes direct killing of the infected cells (8, 17), other ligands, such as the ligand found on MCA-induced sarcoma, cause indirect killing by cytokine secretion (14). Notably, the ligand on MCA-transformed cells undergoes immune editing by NKp46/NCR1 pressure (14), whereas the ligand on D122 cells described in this study apparently does not. Finally, we have recently shown that a ligand for NKp46/NCR1 is expressed by human and mouse β cells (27, 28), and it was also demonstrated that upon human CMV infection of dendritic cells, an unknown ligand of NKp46/NCR1 is downregulated to prevent NK cell-mediated lysis of the infected dendritic cells (30–33).

This situation of numerous ligands for an NK activating receptor is not unusual, as other NK activating receptors also interact with various proteins. For example, human NKG2D recognizes eight different ligands (MICA, MICB, and ULBP1–6) (34), and NKp30 recognizes the pp65 protein of human CMV (35), BAT3 (36), B7-H6 (37), the HA of pox viruses (29), and another unknown ligand expressed by dendritic cells (33). It is possible that each NK activating receptor recognizes various ligands to enable the detection of a wide spectrum of tumors and pathogens via the usage of a limited number of receptors.

NKp46/NCR1 was previously shown to control the primary tumor growth in several models (13). In this study, we demonstrate that the growth rate of both B16 and D122 cells was not affected by the absence of NKp46/NCR1, but metastases formation was. This result is surprising because we did not detect differences in the tumor cells that were harvested from primary tumors or from metastases, either in their biochemical properties, in the expression level of the NKp46/NCR1 ligand, or in their sensitivity to NK cell killing. Several explanations might account for the NCR1 dependency of metastases, but not of primary tumors. In the process of spontaneous metastasis, micrometastases disseminate from the primary tumor and migrate to their target organs, in this case the lungs. This is a gradual process, in which only few tumor cells are shed from the primary tumor. We hypothesize that in these specific conditions, NK cells are able to kill these few tumor cells in an NKp46/NCR1-dependent manner (thus the differences observed in Fig. 3B and 3C). However, when the mouse is inoculated with a larger amount of cells at a certain time point, such as in the injection of 1×10^5 cells intrafootpad for the generation of the primary tumors in our models, or in the experimental metastasis model we present in Fig. 7, the amount of tumor cells is too large for the NK cells to control at once. Alternatively, it is also possible that NKp46/NCR1 inhibits metastases detachment from the primary tumors, or that it kills the disseminating metastases on their way to the lungs. Additionally, it is possible that the location of the primary tumors is important, as in previous models the cancer cells were s.c. injected into the flank and NK cells migrated to these tumors (12–14), whereas here tumor cells were injected into the footpad to enable spontaneous metastasis. Finally, it is possible that the circulating tumor cells transiently changed their properties during their migration to the lungs and that this is NCR1-dependent.

We still do not know why the absence of NCR1 had a greater effect on D122 cells as compared with the B16 cells. The survival of mice deficient for the NKp46/NCR1 was significantly worse than that of the WT mice in both the B16 and D122 models, but to a much larger extent in the D122 than in the B16 model. In contrast, the CD107a degranulation and direct killing of peripheral blood NK cells taken from immunocompetent mice was significantly better compared with the KO cells for both the B16 and D122 cells. It is possible that the absence of NCR1 also affects other parameters that are not associated with direct NK cell killing.

The observations presented in this study also have clinical implications. The involvement of NKp46/NCR1 in metastasis opens a new field of investigation in which the expression of the NK killer receptors can be manipulated for the control of tumor formation. Manipulation of NKp46 expression might be particularly important since it was demonstrated that at a certain stage of tumor development the NKG2D ligands are actually beneficial for tumor growth (38). In contrast, such tumor-beneficial activity was not demonstrated with regard to NKp46/NCR1. Therefore we suggest that enhancing NKp46/NCR1 activity either through the elevation of its expression or by cytokine activation might be beneficial for the treatment of tumor metastasis.

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Disclosures

The authors have no financial conflicts of interest.

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