**ABSTRACT**

CD226 is an activating receptor expressed on natural killer (NK) cells, CD8+ T cells, and other immune cells. Upon binding to its ligands expressed on target cells, CD226 activates intracellular signaling that triggers cytokine production and degranulation in NK cells. However, the role of CD226 in contact dynamics between NK and cancer cells has remained unclear. Our time-lapse images showed that individual wild-type CD226+ NK cells contacted B16F10 melanoma cells for 23.7 min, but Cd226−/− NK cells only for 12.8 min, although both NK cell subsets showed equal contact frequency over 4 h. On the surface of B16F10 cells, CD226+ cells stayed at the same site with oscillating movement (named stable contact), while Cd226−/− NK cells moved around at a velocity of 4 μm/min (named unstable contact). Consequently, Cd226−/− NK cells did not kill B16F10 cells in vitro and did not inhibit their metastasis into the lung in vivo. Taken together, our data demonstrate that CD226 enables prolonged stable interaction between NK and cancer cells, which is needed for efficient killing of cancer cells.

**Keywords:** Contact duration; contact dynamics; contact stability; melanoma; NK cell-mediated cytotoxicity

**Introduction**

Natural killer (NK) cells kill virus-infected and cancer cells while sparing autologous normal cells.1,2 NK cell cytotoxicity is regulated by the balance of activating and inhibitory signals received at the immunological synapse.3 The inhibitory signals are generated by the binding of MHC-I molecules on target cells to killer cell immunoglobulin-like receptors in humans, Ly49 in mice, and a CD94–NK group 2 member A (NKG2A) heterodimer in both species.3–5 NK cells sense signs of infection and malignant transformation through the activating receptors NKG2D, natural cytotoxic receptor, 2B4, and CD226 (also called DNAM-1).3,6 Activating receptors facilitate the conjugation of NK and cancer cells, and transduce signals that augment cytokine production by and degranulation of NK cells. The lack of inhibitory receptors reduces NK cell responsiveness by a process called NK cell education.7 Educated NK cells form stable conjugates with cancer cells via inside-out signaling to LFA-1 by activating receptors.7 Educated NK cells also display the coordinated regulation and colocalization of CD226 and LFA-1 at the immunological synapse, which are essential for tumor cell killing by educated NK cells.8

CD226 is a member of the immunoglobulin superfamily and is expressed on the majority of NK cells, T cells, and monocytes.9–11 CD155 (poliovirus receptor) and its family member CD112 (also called nectin-2) are ligands for human and mouse CD226.6,12,13 Upon ligation, Src kinase phosphorylates the cytoplasmic domain of CD226, which triggers the binding of the adaptor Grb2, activation of the Vav-1, phosphatidylinositol 3′ kinase, phospholipase C-γ1, and protein kinases Erk and Akt, as well as calcium influx.23 This signaling cascade increases actin polymerization and granule polarization, which is required for cytotoxic killing of target cells, and also augments IFN-γ production by NK cells.23 Anti-CD226 neutralizing antibodies inhibit NK cell cytotoxicity.
against many cancer cells expressing its ligand. CD226 deficiency in mice increases susceptibility to carcinogen-induced fibrosarcoma and papilloma. CD226 is also implicated in NK cell–mediated elimination of HIV-infected CD4+ T cells and plays a critical role in the expansion and maintenance of virus-specific memory NK cells in mouse cytomegalovirus–infected mice. Overall, these data suggest that CD226 promotes NK cell activation, at least in part, by activating intracellular signaling for survival, cytokine production, and cytotoxicity. CD226-deficient CD8+ T cells weakly conjugate with tumor cells and show a decreased recruitment of LFA-1 and lipid rafts to the immunological synapse, which correlates with reduced tumor cell killing in vitro, although these cells show normal production and degranulation of cytotoxic granules after T cell receptor stimulation. CD226 is also critical for the ability of CD8+ T cells to eliminate tumor cells during anti-TIGIT antibody therapy.

In addition, CD226 is able to act as an adhesion molecule: CD226-transfected COS-7 cells bind Colo-205 cells, and a CD226-expressing NK cell line (YT-S) conjugates with CD155-expressing RMA-S cells. Yet, several questions remain. How many times does each CD226+ NK cell contact cancer cells? How long do such contacts last? Does CD226 affect contact stability? Do all CD226+ NK cells contact cancer cells? To address these issues, we examined the contact dynamics between CD226-deficient NK and cancer cells at a single-cell level using time-lapse imaging. Our data show that CD226-deficient NK cells fail to establish long-lasting stable contacts with cancer cells and have impaired antitumor activity in vitro and in vivo.

Results

**Cd226−/− NK cells show impaired cytotoxicity at the population level**

Because NK cell cytotoxicity is largely regulated by interactions between activating receptors on NK cells and their respective ligands on cancer cells, we first examined the surface expression of activating receptors on NK cells. All effector NK cells were NK1.1+, LFA-1+, NKG2D+, and CD69+, but only half of them were CD226+ (Fig. 1A). Wild-type (WT) and Cd226−/− NK cells showed similar expression patterns except for CD226 (Fig. 1B). Target B16F10 cells expressed CD155 (CD226 ligand) but not ICAM-1 (LFA-1 ligand), Rae-1, H-2Kb, or MULT-1 (NKG2D ligands) (Fig. 1C), suggesting that the B16F10 cell line is a good tool to study the role of CD226 in the recognition of cancer cells by NK cells because it allows ruling out the interference from other receptors. In the in vitro LDH assay, WT NK cells destroyed B16F10 target cells better than did Cd226−/− NK cells.

![Figure 1. Cd226−/− NK cells show impaired cytotoxicity.](image-url)
NK cells (Fig. 1D). When co-cultured with B16F10 cells, WT NK cells showed higher CD107a expression (degranulation marker) than did CD226−/− NK cells (Fig. 1E). However, WT and CD226−/− NK cells had similar amounts of intracellular cytotoxic proteins, such as perforin and granzyme B (Fig. 1F).

We also verified the role of CD226 in NK cell cytotoxicity by using purified CD226WT NK cells (Fig. 2A). These cells showed higher cytotoxicity against B16F10 cells (Fig. 2A) and better degranulation (Fig. 2B) than did WT CD226−/− NK cells. Neutralizing antibody against CD226 decreased WT NK cell cytotoxicity against B16F10 cells, but anti-NKG2D antibody did not (Fig. 2C). WT NK cells only weakly killed B16F10 cells transfected with CD155 siRNA compared with B16F10 cells transfected with negative-control siRNA (Fig. 2D). These data suggest that CD226 deficiency in NK cells impairs degranulation and cytotoxicity without affecting the levels of intracellular cytotoxic molecules.

**Cd226−/− NK cells show impaired cytotoxicity at the single-cell level**

We also assessed the role of CD226 in NK cell cytotoxicity at the single-cell level by using time-lapse imaging. We mixed calcine AM-stained B16F10 cells and unsorted (Movie S1), CD226+ (Movie S2), or CD226− (Movie S3) WT NK cells or CD226−/− NK cells (Movie S4). In addition, we mixed unsorted WT NK cells and calcine AM-stained B16F10 cells transfected with CD155 siRNA (Movie S5). Representative images collected at 2-h intervals are shown in Fig. 3A. B16F10 cells appeared sessile and extended and retracted pseudopods; these cells grew well and attached to dishes. Upon encountering NK cells, B16F10 cells detached and became rounded (called rounding phase), followed by the uptake of PtdIns (called PtdIns uptake phase). Unsorted and CD226+ WT NK cells efficiently killed B16F10 cells, but CD226− WT NK cells and CD226−/− NK cells did not. In addition, unsorted WT NK cells weakly killed B16F10 cells transfected with CD155 siRNA (Fig. 3A and B). In the presence of unsorted or CD226+ WT NK cells, B16F10 cells became rounded within 29–33 min (Fig. 3C) and were stained with PtdIns within further 92–102 min (Fig. 3D). Only a few B16F10 cells died in the presence of CD226− WT NK cells or CD226−/− NK cells although their dying kinetics was similar to those in the presence of unsorted or CD226+ WT NK cells. In addition, only a few B16F10 cells transfected with CD155 siRNA died in the presence of total WT NK cells.

Next, we analyzed NK cell behavior. NK cell motility could be divided into 2 stages: search and contact (Fig. 4A). WT and CD226−/− NK cells similarly migrated at approximately 5 μm/min during the search stage (Fig. 4B) and at <2 μm/min during the contact stage (data not shown). Both WT and CD226−/− NK cells moved in all directions and showed similar track lengths (Fig. 4C), straightness scores (Fig. 4D), and mean square displacement (Fig. 4E), which indicates long-distance random migration of NK cells at the searching stage. These data suggest that CD226 does not affect NK cell migration.

**Cd226−/− NK cells show short unstable contacts with B16F10 cells**

We next analyzed the contact modes of NK and B16F10 cells. We found 3 types of NK cells: (1) cells that moved freely without attraction to B16F10 cells; (2) cells that contacted and killed B16F10 cells; (3) cells that contacted B16F10 cells but did not
kill them (Fig. 5A). Based on these differences, we analyzed contact dynamics of WT and Cd226−/− NK cells. WT and Cd226−/− NK cells contacted B16F10 cells at equal frequencies (1.3 times in 4 h, data not shown). The ratios of contacting cells per total number of cells were also similar: 19% for unsorted WT NK cells, 18% for purified CD226+ WT NK cells, 22% for purified CD226− WT NK cells, and 21% for unsorted WT NK cells with CD155 siRNA-transfected B16F10 cells (Fig. 5B). Nevertheless, only a few NK cells contacted cancer cells in all NK cell subsets; the outcomes of the contacts were markedly different depending on the presence of CD226: 80% of contacts with purified CD226+ NK cells but only 3% of contacts with purified CD226− NK cells led to the death of B16F10 cells (Fig. 5C). In addition, 5% of contacts with Cd226−/− NK cells killed B16F10 cells and 15% of contacts with unsorted WT NK cells killed B16F10 cells transfected with CD155 siRNA (Fig. 5C). Contact duration between cancer cells and NK cells differed: it was 23.3 min for most unsorted WT cells and 23.7 min for most CD226+ WT NK cells; 10.8 min for most CD226− WT cells and 12.8 min for most Cd226−/− NK cells (Fig. 5D and E). These data indicate that CD226 prolongs the duration of contact between NK and B16F10 cells but does not affect their contact frequency. These data indicate that CD226 prolongs the duration of contact between NK and B16F10 cells but does not affect their contact frequency.

We also analyzed contact stability. Some NK cells showed oscillating movement on the surface of B16F10 cells at an average velocity of < 2 μm/min (called stable contact), while others were moving around on the surface of target cells at a speed of 4 μm/min (called unstable contact) (Fig. 6A and Movie S6). More unsorted or CD226+ WT NK cells showed stable contacts than did CD226− WT NK cells or Cd226−/− NK cells. In addition, unsorted WT NK cells showed unstable contacts with B16F10 cells transfected with CD155 siRNA (Fig. 6B). Our binding assay confirmed weaker binding of Cd226−/− NK cells than WT NK cells (Fig. 6C). Overall, these data suggest that CD226 enhances binding of NK cells to B16F10 cells and improves contact stability.

**Cd226−/− NK cells only weakly inhibit tumor metastasis in vivo**

Because of shorter and unstable contacts, we postulated that Cd226−/− NK cells might have low anti-metastatic activity in vivo. When injected intravenously into recipient mice,
B16F10 cells formed 135 metastatic colonies on the surface of lung at 2 weeks after transfer (Fig. 7A and B). WT NK cells inhibited metastasis by 83%, whereas \( \text{Cd226}^{-/-} \) NK cells inhibited it by 28% (Fig. 7A and B). On histological assessment, WT NK cells efficiently inhibited the formation of tumor colonies in the deeper areas of the lung compared with the control group, but \( \text{Cd226}^{-/-} \) NK cells weakly inhibit tumor metastasis to the lung (Fig. 7C). These data suggest that \( \text{Cd226}^{-/-} \) NK cells have weak anti-metastatic activity in vivo, probably due to the impairment of their interaction with cancer cells.
Discussion

Our study provides several insights into the cytotoxic mechanisms of NK cells. We found 3 CD226-independent events: (i) CD226 does not affect the speed of NK cell migration (≈5 μm/min); (ii) CD226 does not affect the frequency of NK cell contacts with cancer cells; and (iii) a small fraction (approximately 20%) of NK cells randomly contact cancer cells even if the former do not express CD226. In contrast, we found that (i) CD226 prolongs the duration of contacts between NK and cancer cells, (ii) it increases contact stability; (iii) it enhances the antitumor activity of NK cells in vitro and in vivo. Collectively, our data reveal how NK cells kill cancer cells in a CD226-dependent manner.

Our study extends the previous data on the dynamics of cancer cell killing by NK cells. NK cells kill target cells in a well-ordered manner: transport of the lytic granules to the microtubule-organizing center; polarization of the organizing center to the immunological synapse; and exocytosis of lytic granules into the immunological synapse.29-33 The released perforin and granzymes induces membrane protrusions in the target cells and their apoptotic and necrotic cell death.34

![Figure 6](image)

**Figure 6.** Stability of contacts between NK and cancer cells. (A) Unstained NK cells (unsorted WT, CD226 WT, CD226 WT, or Cd226–/– NK cells) and calcein AM–stained B16F10 cells (transfected with CD155 siRNA or not) were imaged every 2 min from 1 h to 5 h. Representative images of stable and unstable contacts (magnification, 200 ×; electronically zoomed; scale bar, 15 μm; n = 10 movies of 3 independent experiments per group). (B) Ratios of stable and unstable contacts between cancer cells and NK cells (n = 142, 84, 116, 111, and 146 from the left). (C) Binding rates of cancer cells and wild-type or Cd226–/– NK cells analyzed by flow cytometry (n = 3, mean ± SEM, *p < 0.01).

![Figure 7](image)

**Figure 7.** Cd226–/– NK cells weakly inhibit the lung metastasis of cancer cells in vivo. C57BL/6 mice (n = 6) were injected intravenously with B16F10 cells on day 0 and NK cells on day 2. Lungs were collected on day 14 and the metastatic nodules were counted. Mean ± SEM (A) and representative images are shown (B). Representative images of hematoxylin and eosin staining of lung sections (magnification, ×100) (C). *p < 0.01.
dynamics of killing by NK cells has also been reported: NK cells can deliver the lytic hit within 10 min,\textsuperscript{29,35,36} individual NK cells sequentially kill up to 6 target cells over 16 h.\textsuperscript{37} NK cells are able to kill more target cells if the cytotoxic granules are recycled\textsuperscript{38,39}; eventual depletion of perforin in the granules is thought to contribute to the exhaustion of the NK cell killing capacity. In this study, we examined the dynamics of the NK cell contacts with cancer cells that precede killing of the latter. We found that if NK cells stably contact cancer cells for more than 23 min at a frequency of 2–3 per 4 h, they can efficiently kill cancer cells, and this ability depends on CD226 expressed on the NK cell surface.

Our data confirm the critical role of CD226 in cancer cell killing by NK cells. CD226 controls immunological synapse formation, cytotoxicity, cytokine secretion, and differentiation of NK cells.\textsuperscript{3} Our data show that CD226 also ensures the sufficient contact of NK and cancer cells required for efficient killing of cancer cells. In contrast, several studies suggest that CD226 does not directly affect NK cell functions. Anti-CD226 neutralizing antibodies do not alter cytokine production by NK cells activated by IL-12 and IL-18.\textsuperscript{2} However, it has not been tested whether these antibodies inhibit cytokine production by NK cells activated by CD226 ligands expressed on cancer cells. NK cells with mutated CD226 (Y319F and N321Q) conjugate with cancer cells normally, although these NK cells cannot kill cancer cells.\textsuperscript{23} However, since contact duration and stability were not examined in the latter study, it is difficult to conclude whether the CD226 mutations directly affected contact quality. Overall, the available information suggests that CD226 ensures sufficient contact of NK and cancer cells and its downstream signaling is obviously required for degranulation of perforin and granzymes and cancer cell killing.

It is becoming clear that considerable functional heterogeneity exists within the peripheral NK cells.\textsuperscript{40} Most NK cells (approximately 75%) are unable to kill cancer cells, whereas a minority of NK cells are responsible for most cancer cell death, and serial killers killing more than 3 cancer cells were observed in 12-h imaging experiments.\textsuperscript{41} However, the latter study did not characterize the phenotypes of killer and non-killer NK cells. Our data consistently suggest that a small fraction of NK cells (approximately 20%) are responsible for most cancer cell death. Furthermore, we suggest that CD226 might be one of the markers of killer NK cells. NK cells of various maturation stages exhibit differential responsiveness to cytokines and different levels of cytotoxicity and are found in various tissues, such as the spleen, lymph nodes, liver, and lung.\textsuperscript{32} CD226 is expressed in all NK cell progenitors, whereas it is downregulated as NK cells mature, generating CD226\textsuperscript{+} and CD226\textsuperscript{−} NK cells in the periphery.\textsuperscript{2} Following stimulation with IL-12 and IL-18, CD226\textsuperscript{+} NK cells produce more IFN-\textgamma, IL-6, CCL5, and GM-CSF than do CD226\textsuperscript{−} NK cells.\textsuperscript{2} CD226\textsuperscript{+} NK cells also suppress tumor growth and metastasis in vivo more effectively than CD226\textsuperscript{−} NK cells.\textsuperscript{5} Our data provide evidence that half of the splenic NK cells were CD226\textsuperscript{+} NK cells and that CD226\textsuperscript{−} have better antitumor activity than CD226\textsuperscript{−} NK cells.

Further studies will be required to address the cytotoxic mechanisms of CD226-deficient NK cells. It will be interesting to study any compensatory signals in these cells. The CD155 and CD112 ligands expressed on cancer cells can bind not only the activating receptor CD226 but also the inhibitory receptors T cell immunoreceptor with Ig and ITIM domain (TIGIT) or CD96 on immune cells.\textsuperscript{53} Thus, the loss of CD226 would increase the availability of CD155 and CD112 for TIGIT, which might complicate the exact interpretation of the functional data for CD226\textsuperscript{−} NK cells. Interestingly, we observed that CD226\textsuperscript{−} NK cells expressed TIGIT and killer cell lectin-like receptor G1 (KLRG1) at much higher levels than did CD226\textsuperscript{+} NK cells (data not shown). In addition, a total of 561 and 305 genes were found to be over- and under-expressed, respectively, in CD226\textsuperscript{+} NK cells compared with CD226\textsuperscript{−} NK cells.\textsuperscript{2} Thus, we cannot exclude that weak cytotoxicity and physical contacts of CD226\textsuperscript{−} NK cells might be due to the enhanced expression of TIGIT, KLRG1, or other unknown factors. Our future study will help to reveal how NK cells gather and balance positive and negative information from cancer cells.

It will be interesting to examine why CD226\textsuperscript{−} NK cells have weak antitumor activity in vivo. Their failure to control lung metastasis could be due to impaired contacts with cancer cells, impaired homing to the tumor site, or decreased viability. It was previously reported that treatment of endothelial cells with anti-CD155 antibody or treatment of monocytes with anti-CD226 antibody prevented transendothelial migration of monocytes, although this was shown in an in vitro system.\textsuperscript{44} These data indirectly suggest that CD226\textsuperscript{−} NK cells might show impaired homing to the tumor site. The data from our preliminary immunofluorescence study show that more Nkp46\textsuperscript{+} NK cells are present in the lung metastatic foci of mice injected with WT NK cells than in those of mice injected with PBS or CD226\textsuperscript{−} NK cells (data not shown). These data will help to reveal in greater detail why CD226\textsuperscript{−} NK cells show impaired control of tumor metastasis in vivo.

It will also be interesting to study whether CD226 is the main activating receptor in NK cells. NK cells simultaneously use several activating receptors, such as CD226 recognizing CD155 and CD112, LFA-1 binding to ICAM-1, and NKG2D binding to Rae-1 and MULT-1.\textsuperscript{45,46} Our unpublished observations suggest that CD226-deficient NK cells normally bind and kill C1498 and Yac-1 cells expressing ICAM-1, CD155, Rae-1, and MULT-1. Thus, we postulate that CD226-deficient NK cells can use other activating receptors, such as LFA-1 and NKG2D, to kill cancer cells. This study was designed with a very narrow scope: to examine the role of CD226 in contact dynamics of NK and cancer cells. To address this issue, we used CD226-deficient NK cells as effector cells and B16F10 as target cells; B16F10 selectively express CD155 but not ICAM-1, Rae-1, or MULT-1. Thus, it is difficult to conclude from our data whether CD226 is the main activating receptor in NK cells. Our next study will examine the possible hierarchy of several activating receptors in NK cell activation to reveal how NK cells gather positive information from cancer cells.

It will also be worth comparing the contacts between dendritic cells and CD226\textsuperscript{+} or CD226\textsuperscript{−} NK cells. The proliferation and production of IFN-\textgamma and TNF-\alpha of CD226-deficient CD8\textsuperscript{+} T cells was reduced when stimulated with dendritic cells, although it was normal when stimulated with anti-CD3 and anti-CD28 antibodies.\textsuperscript{27} CD226\textsuperscript{−} NK cells would have access to dendritic cells given the role of CD226 in the functioning of the immunological synapse and generation of effector cytokines.
CD226<sup>−</sup> NK cells may be positioned to alert other leukocytes through secretion of MIP-1 in a non-synapse-dependent but cytokine-dependent manner. Presumably, these secretion events occur after differentiation of CD226<sup>+</sup> NK cells into CD226<sup>−</sup> NK cells. It will be important to assess the behavior of these CD226<sup>+</sup> NK cells in the presence of dendritic cells in vitro and in vivo.

Our studies might have implications for the rational design of NK cell–based immunotherapy of cancer patients. Up-regulation of activating ligands on tumor cells is also a good strategy to improve antitumor activity of NK cells. Several studies reported that anticancer drugs such as inhibitors of proteasome, histone deacetylase, GSK3, and HSP-90 as well as genotoxic drugs increase surface expression of NKG2D and CD226 ligands on cancer cells.<sup>47-49</sup> Treatment of multiple myeloma with nitric oxide donors increases the expression of CD155 on cancer cells, rendering these cells more susceptible to NK cell–mediated killing.<sup>48</sup> Treatment of K-562, HCT-116, and Hep-G2 cells with cytochalasin D, nocodazole, or docetaxel increases cell surface expression of NKG2D and CD226 ligands on cancer cells.<sup>50</sup> Treatment of multiple myeloma with nitric oxide donors increases the expression of CD155 on cancer cells, rendering these cells more susceptible to NK cell–mediated killing.<sup>50</sup>

Materials and methods

Mice and cells

CD226<sup>−/−</sup> mice were provided by Dr. A. Shibuya (University of Tsukuba, Ibaraki, Japan) and C57BL/6 mice were purchased from Samtako (Gyeonggi, Korea). All animal studies were approved by the Chungbuk National University Animal Experimentation Ethics Committee and were performed in accordance with the approved guidelines. NK cells were isolated from mouse spleen cells by negative selection using an NK iso- lation kit (Milltenyi Biotec, Auburn, CA, USA). Purified NK cells were cultured in RPMI 1640 medium supplemented with 3,000 U/ml recombinant human IL-2 (Bayer HealthCare Pharmaceuticals, Emeryville, CA, USA), 10% fetal bovine serum, 3,000 U/ml recombinant human IL-2 (Bayer HealthCare Pharmaceuticals, Emeryville, CA, USA), 10% fetal bovine serum, 50 µM 2-mercaptoethanol. Cell purity exceeded 90%.

IL-2-activated NK cells were used from day 10 to 12. Activated NK cells were further sorted into CD226<sup>+</sup> and CD226<sup>−</sup> cells using a FACSaria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Purity of CD226<sup>−</sup> cells exceeded 90%. B16F10 melanoma cells were purchased from American Type Culture Collection (Manassas, VA, USA).

Flow cytometry

Cells were stained for 15 min at 4°C with antibodies against mouse CD3, CD69, ICAM-1, H-2K<sup>+</sup>, LFA-1, NK1.1, NKG2D (BD Biosciences), CD226, CD155 (BioLegend, San Diego, CA, USA), or Rae-1 (R&D Systems, Minneapolis, MN, USA). NK cells were also fixed using a CytoFix-CytoPerm Kit (BD Biosciences) according to the manufacturer’s instructions and then were stained with anti-perforin–APC antibody and anti-gran- yme B–FITC antibody (ebioscience, San Diego, CA, USA). To analyze exocytosis, NK cells (1 × 10<sup>5</sup>) were mixed with B16F10 cells (1 × 10<sup>5</sup>), centrifuged at 1,000 rpm for 1 min, and incubated for 2 h at 37°C in the presence of anti-CD107a–FITC antibody (BD Biosciences). The cells were then stained with anti-NK1.1–APC antibody. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and the data were processed using Cell Quest Pro software (BD Biosciences).

Lactate dehydrogenase (LDH) release assay

NK cells were incubated with B16F10 cells in 96-well plates at various effector-to-target cell ratios. After 4-h incubation, the plates were centrifuged and 100 µl of the supernatants was transferred to new 96-well plates. B16F10 cell death was determined using LDH release assay according to the manufacturer’s instructions (Takara, Shiga, Japan). The percentage of specific lysis was calculated from LDH content as follows: (experimental release – target spontaneous release) / (target maximum release – target spontaneous release) × 100%.<sup>51</sup>

RNA interference

A double-stranded small interfering RNA (siRNA) oligonucleotide targeting CD155 (GenBank accession number NM_027514), 5’-GAG CAU AAA GCA AGG UUG AdTdT-3’, 5’-CCA CUG CAC UUU UCU AGG UdTdT-3’, 5’-CUA GGC UAC UUU CUU AdTdT-3’, was chemically synthesized (Bioneer, Daejon, Korea) and transfected into B16F10 cells (25 pmol per well of a 6-well plate) using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Cells were incubated with the siRNA–lipid complex in growth medium without antibiotics for 48 h. Control cells were transfected with a negative control siRNA oligonucleotide at a matching concentration.

Time-lapse imaging

B16F10 cells (5 × 10<sup>4</sup> cells/35 mm dish) were pre-cultured in serum-containing medium for 4 h at 37°C, washed twice with serum-containing medium, and stained with 1 µM calcein acetoxymethyl ester (Calcein AM, Thermo Fisher Scientific) in serum-free medium for 30 min at 37°C, and washed twice. NK
cells (1 × 10⁵ cells/35 mm dish) were then added to calcein AM–labeled B16F10 cells (target) in 35 mm dish. Propidium iodide (PtdIns, 2 μM) was added to medium. Time-lapse imaging was performed with a Biostation IM-Q microscope equipped with a 20 × magnification objective (numeric aperture 0.5) in an environmental chamber kept at 37°C and 5% CO2 (Nikon Inc., Melville, NY, USA). Dishes were precoated for 1 h in the chamber and images were acquired every 2 min for 4 h.⁵¹ NK cells were manually tracked by using Imaris software version 7.2 (Bitplane, Zurich, Switzerland). Instantaneous velocity was calculated automatically by Imaris software.

The number of contacts that lasted for >6 min was determined. PtdIns-stained and calcein AM–leaking B16F10 cells were considered dead cells.

### Cell binding assay

NK cells (1 × 10⁶ cells/ml) were stained with 0.5 μM 5-chloromethylfluorescein diacetate (CMFDA, Thermo Fisher Scientific) and B16F10 cells (1 × 10⁵ cells/ml) with 5 μM 5-(and-6)-(4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTPX, Thermo Fisher Scientific) in serum-free medium for 15 min at 37°C. After staining, cells were washed twice in culture medium with 10% fetal bovine serum. NK cells (4 × 10⁵) and B16F10 cells (1 × 10⁵) were mixed in a 12 × 75-mm polystyrene tube (BD Biosciences) and centrifuged at 1,000 rpm for 1 min, and pellets were incubated at 37°C for 30 min. Cell mixtures were then gently suspended and analyzed by flow cytometry. The conjugation ratio was calculated as the portion of CMFDA/CMTPX double-positive events within the CMTPX-positive events.

### In vivo metastasis model

B16F10 cells (4 × 10³ cells/mouse) were injected intravenously into C57BL/6 mice on day 0. NK cells (4 × 10⁶ cells/mouse) were injected intravenously on day 2. Lungs were collected on day 14 and the metastatic nodules were counted.⁵⁵ For histologic analysis, lung tissues were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 5 μm thickness. Sections were stained with hematoxylin and eosin.

### Statistical analysis

Data represent the mean ± SEM of at least 3 independent experiments performed in triplicates (in vitro) or 6 mice (in vivo); p values were calculated using Student’s t-test or one-way ANOVA in GraphPad Prism Software (San Diego, CA, USA). For NK or B16F10 cell counts, p values were calculated using Mann–Whitney test in GraphPad Prism Software.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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