Supporting Information

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SI Materials and Methods

Natural Killer Cytotoxicity Assay. Natural killer (NK) cytotoxicity assay was performed as previously described (1). In brief, target cells were labeled with $Na_2{}^{51}CrO_4$ (PerkinElmer) at 37 °C for 1 h. Then, target cells were incubated with effector cells in 96-well round-bottom plates at indicated effector/target (E/T) ratios for 4 h. The percentage of specific ^{51}Cr release was calculated as follows: 100 × (experimental lysis − spontaneous lysis)/(maximal lysis − spontaneous lysis).

In Vitro NK Cell Death Assay. IL-2–activated NK cells (1×10^5 per well) were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (1 μM; Invitrogen), and then cocultured with target cells in indicated effector/target (E/T) ratios in 96-well round-bottom plates for 2 h at 37 °C. After coculture, cells were stained with anti-NK1.1 mAb (PK136; BioLegend) and propidium iodide (PI; Sigma). The percentage of PI-positive cells within the $CFSE^+$ $NK1.1^+$ population was measured on a FACSCanto II (BD Biosciences). For blocking assays, NK cells were pretreated with anti-CD16/CD32 mAb (2.4G2, 10 μg/mL; BD), and then incubated with the following mAb (10 μg/mL) for 30 min before coculture: anti-natural killer type 2 group D (NKG2D) (CX5; generated as previously described) (2), anti-Fas ligand (FasL) (Kay-10; BioLegend), anti-TNF-related apoptosis-inducing ligand (TRAIL) (N2B2; BioLegend), and anti-DNAX accessory molecule 1 (DNAM-1) (10E5; BioLegend).

In Vivo NK Cell Death Assay. CFSE-labeled wild type (WT) C57BL/6 NK cells (5×10^6) were cocultured with RMA or RMA stably expressing retinoic acid early inducible protein 1 δ (RMA/Rae-1δ) (5×10^6 each) for 15 min, and these cells were injected into spleen of recombination activating gene-1-deficient ($Rag\text{-}1^{-/-}$) mice ($n = 4$ each). After 4 h, splenocytes were stained with anti-NK1.1 mAb, and the percentage of $CFSE^+$ $NK1.1^+$ cells in whole splenocytes was calculated. To address the contribution of host NK cells to adoptively transferred NK cell death, CFSE-labeled NK1.1-negaitve BALB/c NK cells (5×10^6) cocultured with RMA or RMA/Rae-1δ (5×10^6 each) were injected into spleen of WT C57BL/6 mice or NK-depleted C57BL/6 mice that had been intraperitoneally injected with anti-NK1.1 mAb (250 μg/mouse) 4 d before the adoptive transfer. At 4 h after the transfer, splenocytes were stained with anti–$H\text{-}2K^d$ mAb (SF1-1.1; Biolegend), and the percentage of $CFSE^+$ $H\text{-}2K^{d+}$ cells in whole splenocytes was calculated.

Stimulation of NKG2D Receptor. Anti-NKG2D mAb (CX5) or control rat IgG were immobilized on 96-well flat-bottom plates by DOTAP (Sigma) at 10 μg/mL as previously described (3). IL-2–activated NK cells (2×10^5 per well) pretreated with anti-CD16/CD32 mAb (2.4G2) were then added onto coated plates and cultured in growth medium with recombinant human IL-2 (100 units/mL) for the indicated periods. Then, NK cells were collected and cell death was analyzed by flow cytometry using PI. The amount of IFN-γ in the culture supernatants was determined with the mouse IFN-γ Femto-HS high-sensitivity ELISA kit (eBioscience), according to the manufacturer's instructions.

In Vitro Trogocytosis Assay. CFSE-labeled, IL-2–activated NK cells (1×10^5 per well) and target cells (1×10^5 per well) were cocultured in 96-well plates for indicated periods at 37 °C. Cells were pretreated with anti-CD16/CD32 mAb (2.4G2), and then stained with allophycocyanin (APC)-conjugated pan-specific Rae-1 mAb (186107; R&D Systems). For the blocking assay using mAb, Fc-blocked cells were pretreated with the following mAb (10 μg/mL) before coculture: anti-NKG2D (CX5), anti-intercellular adhesion molecule-1 (ICAM-1) (KAT-1; eBioscience), anti-CD48 (HM48-1; BioLegend), and anti-2B4 [m2B4 (B6)4458.1; Biolegend]. IgG from rat serum (Sigma) was used as the control antibody. For blocking assays using chemical inhibitors, NK cells were incubated with the following reagents for 30 min before coculture: spleen tyrosine kinase (Syk) inhibitor piceatannol (12.5–100 μM; Wako), PI3-kinase inhibitor LY294002 (12.5–100 μM; Wako), mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) inhibitor U0126 (12.5–50 μM; Wako), Src family kinase inhibitor PP2 (12.5–50 μM; Wako), actin polymerization inhibitor cytochalasin D (20 μM; Sigma), an inhibitor of clathrin-dependent endocytosis chlorpromazine (10 μg/mL; Sigma), an inhibitor of caveolae-dependent endocytosis filipin complex (2 μg/mL; Sigma), and broad endocytosis inhibitor methyl-β cyclodextrin (10 mM; Sigma). For some assays, NK cells were cultured in the hypertonic media containing 0.45 M sucrose (Wako), which inhibits clathrin-dependent endocytosis as described previously (2).

In Vivo Trogocytosis Assay. RMA-S (5×10^5), RMA/Rae-1δ (1×10^6), B16 (5×10^5), or B16/Rae-1ε (2×10^6) were injected s.c. into WT C57BL/6 mice. Tumor tissues were collected at 2 wk after transplantation and treated with collagenase (200 units/mL, Wako). Tumor-infiltrating lymphocytes were stained with anti-DX5 mAb, and anti–Rae-1 mAb, and Rae-1 expression on $DX5^+$ cells was analyzed by flow cytometry. As the other model, RMA or RMA/Rae-1δ (1×10^7 each) were injected into spleen of $Rag\text{-}1^{-/-}$ mice. After 2 h, splenocytes were collected and stained with anti-NK1.1 mAb (PK136; BioLegend) and anti–Rae-1 mAb (R&D Systems). Rae-1 expression on $NK1.1^+$ cells was analyzed by flow cytometry.

Human NK Trogocytosis Assay. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Separate-L (Muto Pure Chemicals) from healthy donors. Mouse pro B-cell line Ba/F3 cells stably expressing a human NKG2D ligand, MHC class I chain-related molecules A (Ba/F3-MICA) was prepared as previously described (2). PBMC were cocultured with Ba/F3-MICA at a 1:1 ratio for 1 h. PBMCs were also cultured with naturally processed soluble MICA (2,000 pg/mL) for 24 h. Then, cells were stained with anti-CD3 mAb (UCHT1, Biolegend), anti-CD56 mAb (HCD56, Biolegend), and anti-MICA/B mAb (6D4, Biolegend). As for the soluble MICA, the supernatant of confluently cultured BaF/3-MICA was harvested, and the concentration was determined by ELISA using Human MICA Duoset (R&D Systems).

RT-PCR. Total RNA was purified from NK cells using Sepasol (Nacalai). Complementary DNAs were synthesized from total RNAs by using oligo(dT) primer (Invitrogen). PCR was performed by using 10-fold serially diluted cDNA templates, AmpliTaq poly (Applied Biosystems). Primer for amplifying *Raet1e* (forward primer 5′-AGA CCT TCC CTG GTG TGA CGT G-3′ and reverse primer 5′-TGT CTG CAT TGG GGT ATG AA-3′) and *Actb* (forward primer 5′-TCC CTG GGG CAG AGT GGT GAA GAT-3′ and reverse primer 5′-ATG GAT GAC GAT ATC GCT GCG CTG-3′) were used.

Confocal Microscopy. For NK cell death analysis, NK cells were labeled with 5- (and 6-) carboxytetramethylrhodamine succinimidyl ester (TAMRA; Invitrogen). After coculture with RMA/Rae-1δ for 2 h, cells were stained with FITC-conjugated annexin V (BD Pharmingen). For trogocytosis analysis, NK cells were stained with biotinylated anti-NK1.1 mAb (PK136; BioLegend), followed by Dylight594-conjugated streptavidin (BioLegend). NK cells were cocultured with RMA/Rae-1δ for 30 min, and then cells were stained with FITC-conjugated, anti–Rae-1 pan-specific mAb (R&D Systems). These cells were then analyzed on a Carl Zeiss, LSM510 confocal laser-scanning microscope with a 63× objective lens.

Flow Cytometric Analysis and Cell Sorting. CFSE-labeled NK cells were pretreated with anti-CD16/CD32 mAb (2.4G2), and then incubated with the following mAb for 30 min at 4 °C before and after coculture: PE-conjugated anti-MULT1 (5D10; eBioscience) PE-conjugated anti–Rae-1γ (CX1; BioLegend), and PE-conjugated anti-NKG2D (C7; BioLegend). Expression of these molecules on NK cells were analyzed by a FACSCanto II flow cytometer (BD Biosciences) after gating on CFSE-positive cells. For cell sorting, CFSE-gated NK cells were purified after 15 min of coculture with tumor cells by an Epics ALTRA cell sorter (Beckman Coulter). $CFSE^+$ NK cells were obtained with more than 98% purity.

Statistical Analysis. For statistical evaluation, a two-tailed, unpaired Student *t* test was used. *P* values <0.05 were considered significant.

1. Cerwenka A, Baron JL, Lanier LL (2001) Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci USA* 98(20):11521–11526.
2. Ogasawara K, et al. (2003) Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity* 18(1):41–51.
3. Ogasawara K, Yoshinaga SK, Lanier LL (2002) Inducible costimulator costimulates cytotoxic activity and IFN-gamma production in activated murine NK cells. *J Immunol* 169(7):3676–3685.

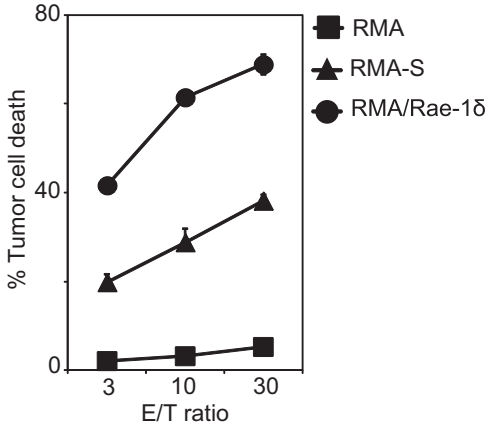

Fig. S1. NK cytotoxicity against RMA, RMA-S, or RMA/Rae-1δ was determined by 4 h ^{51}Cr release assay. Representative data from three independent experiments are shown.

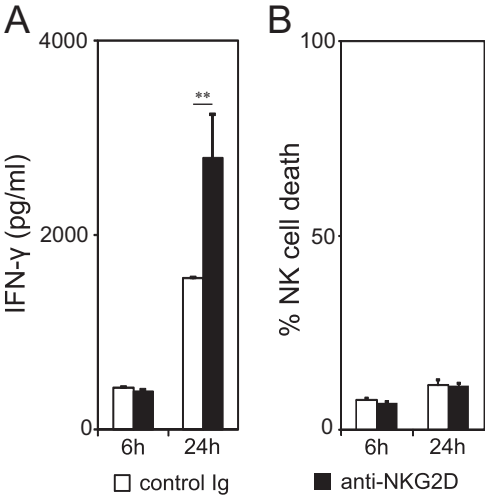

Fig. S2. Cross-linking of NKG2D does not induce NK cell death. IL-2–activated NK cells were cultured on control rat IgG-coated or anti-NKG2D mAb-coated plates for 6 h or 24 h. Production of IFN-γ in cell culture supernatants was measured by ELISA (*A*). The percent NK cell death was quantified by flow cytometry using PI (*B*). Data represent means plus SDs of triplicates. Similar results were obtained in three independent experiments. $^{**}P < 0.01$, compared with control rat IgG.

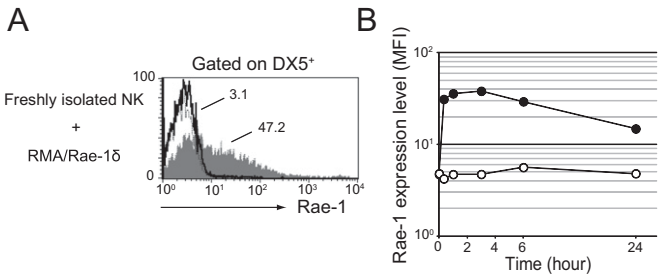

Fig. S3. Trogocytosis of Rae-1 by freshly isolated NK cells. Freshly isolated WT NK cells were cocultured with RMA/Rae-1δ at a 1:1 ratio for 1 h (*A*) or the indicated periods of time (*B*). Acquisition of Rae-1 by DX5$^+$ NK was analyzed as described in Fig. 3 *D* and *E*. Representative data from two independent experiments are shown.

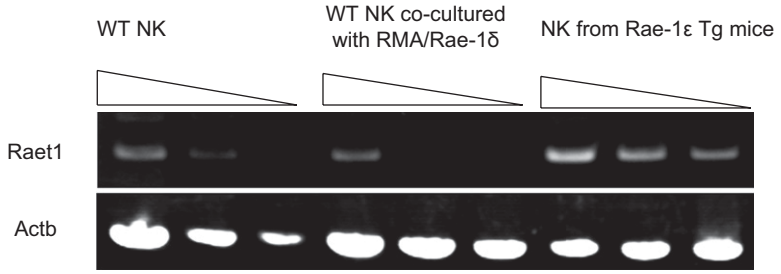

Fig. S4. Expression of Rae-1 transcript in IL-2–activated NK cells. Complementary DNAs were prepared from WT NK cells cultured alone or cocultured with RMA/Rae-1δ, and from Rae-1ε transgenic NK cells. Expression of Rae-1ε and β-actin transcript was analyzed by semiquantitative RT-PCR using 10-fold serially diluted cDNA templates. Representative data from three independent experiments are shown.

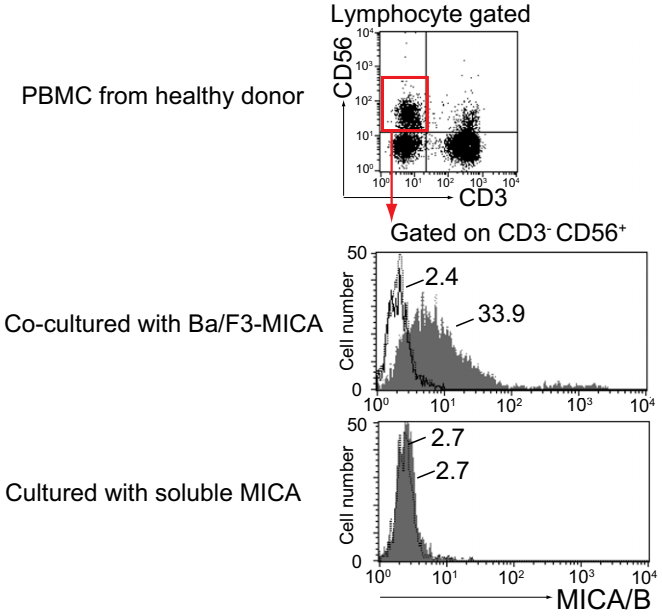

Fig. S5. Human peripheral blood NK cells acquire membrane-bound MICA, but not soluble MICA. Human peripheral blood mononuclear cells were cocultured with Ba/F3 stably expressing MICA (Ba/F3-MICA) or cultured with naturally processed soluble MICA (2,000 pg/mL) for 24 h. Then, acquisition of MICA on CD3$^-$ CD56$^+$ NK cells was analyzed. Dotted line histograms and gray histograms indicate isotype control mAb staining and anti-MICA/B mAb staining, respectively. Solid line indicates anti-MICA/B mAb staining of NK cells cultured alone. Representative data from two independent experiments are shown.

A

Reagent	Inhibitor of	Dose
LY294002	PI3-kinase	12.5-100 μM
Piceatannol	Syk-selective	12.5-100 μM
U0126	MEK/ERK	12.5-50 μM
PP2	Src-family kinase	12.5-50 μM
Cytochalasin D	Actin polymerization	20 μM
Chlorpromazine	Clathrin-dependent endocytosis	10 μg/ml
Filipin complex	Caveolae-mediated endocytosis	2 μg/ml
Methyl-β-cyclodextrin	Broad endocytosis	10 mM

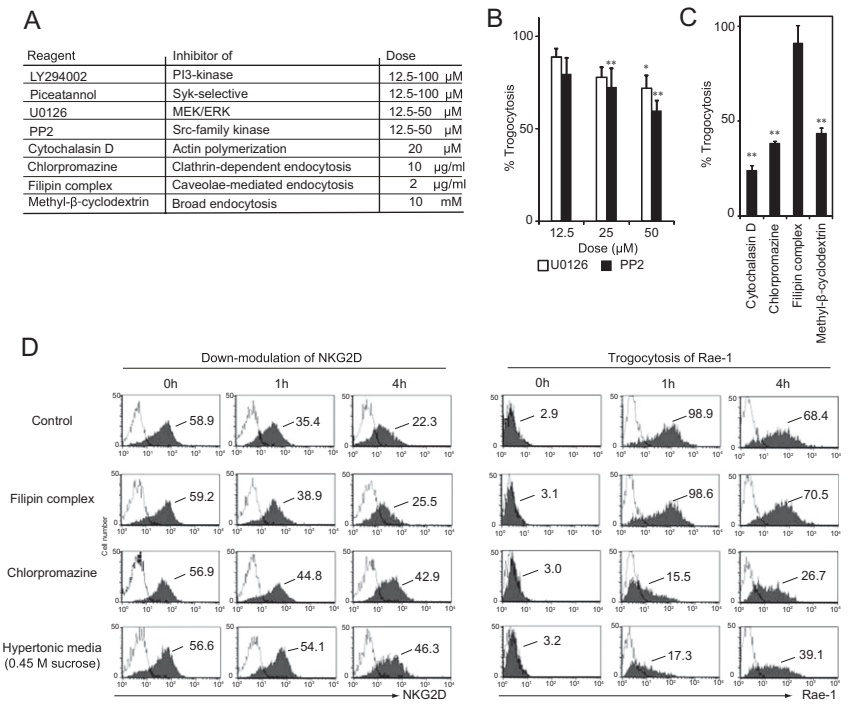

Fig. S6. Both down-modulation of NKG2D and trogocytosis of Rae-1 are mediated through clathrin-dependent endocytosis. (*A*) List of inhibitors used in Fig. 4 and Fig. S6. (*B* and *C*) CFSE-labeled NK cells were cocultured with RMA/Rae-1δ in the presence or absence of the indicated inhibitors for 1 h. Percent trogocytosis of Rae-1 by NK cells was calculated as described in Fig. 4*B*. (*D*) CFSE-labeled NK cells were cocultured with RMA/Rae-1δ in the presence or absence of the indicated inhibitors or in the medium containing 0.45 M sucrose for the indicated periods of time. Dotted line histograms and gray histograms indicate isotype control mAb staining and specific mAb staining, respectively. $*P < 0.05$, $**P < 0.01$, compared with the absence of inhibitor. Representative data from three independent experiments are shown.

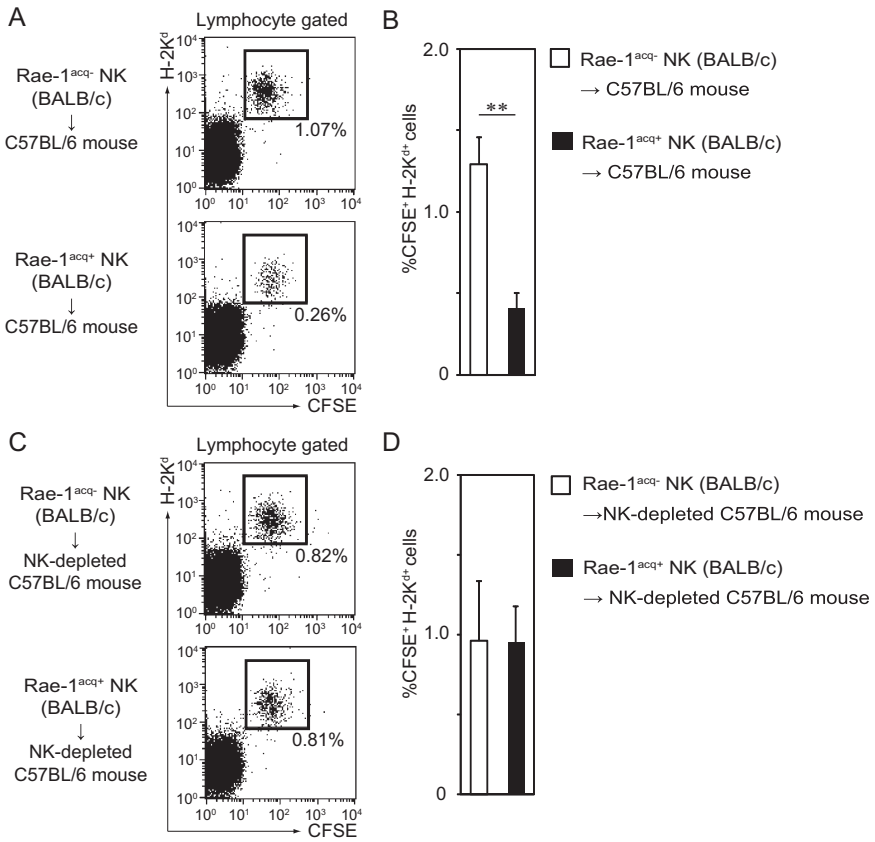

Fig. S7. Rae-1–dressed NK cells are eliminated by other NK cells in vivo. CFSE-labeled BALB/c NK cells were cocultured with RMA or RMA/Rae-1δ at a 1:1 ratio for 15 min. Then, these NK cells were injected into spleen of C57BL/6 mouse ($n = 4$ each) (*A* and *B*) or C57BL/6 mouse that had been injected with anti-NK1.1 mAb to deplete host NK cells ($n = 4$ each) (*C* and *D*). After 4 h, splenocytes were stained with anti–H-2K^d mAb. H-2K^{d+} CFSE$^+$ NK cell population in spleen was analyzed as described in Fig. 5 *A* and *B*. $^{**}P < 0.01$, compared with Rae-1^{acq-} NK cells. Similar results were obtained in two independent experiments.

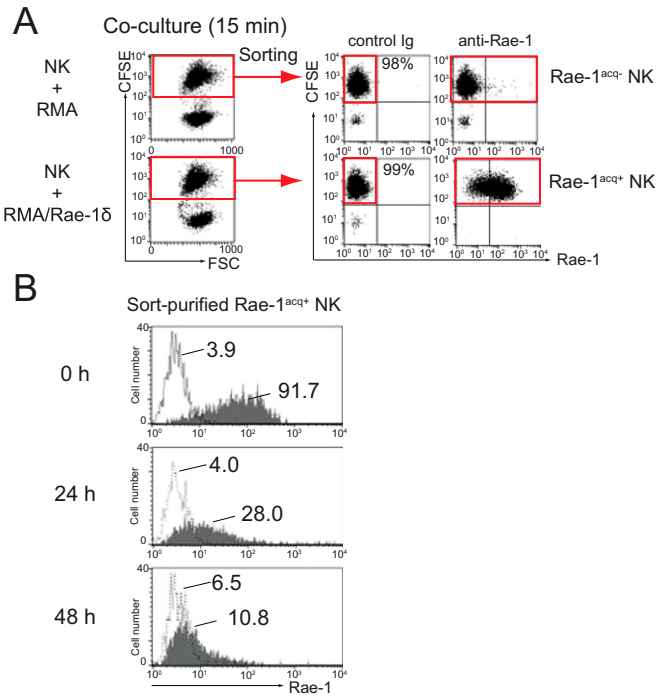

Fig. S8. Sort purification of Rae-1–dressed NK cells. (*A*) Strategy for sorting Rae-1–dressed NK cells. CFSE-labeled NK cells were cocultured with RMA or RMA/Rae-1δ for 15 min at a 1:1 ratio. Then, $CFSE^{+}$ NK cells were purified by cell sorting. Sorted NK cells were stained with anti–Rae-1 mAb or isotype control mAb, and acquisition of Rae-1 was analyzed. (*B*) Stability of Rae-1 on sort-purified NK cells. Rae-1–dressed NK cells were sort purified as described in *A*. After the indicated periods of time, these cells were stained with anti–Rae-1 mAb (gray histograms) or isotype control mAb (dotted line histograms). Representative data from two independent experiments are shown.

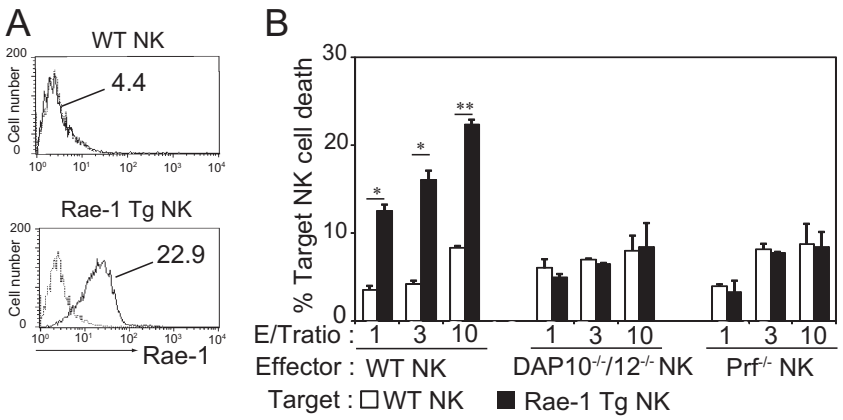

Fig. S9. Rae-1 expression on NK cells is sufficient for NK cell fratricide. (*A*) NK cells from WT mice or Rae-1 transgenic (tg) mice were stained with isotype control mAb (dotted line histograms) or anti–Rae-1 mAb (solid line histograms). (*B*) Target WT NK cells or Rae-1 tg NK cells were labeled with CFSE. These target cells were cocultured with effector NK cells from WT, DNAX-activating protein (DAP) 10 and DAP12 double-deficient ($DAP10^{-/-}/12^{-/-}$), or perforin-deficient ($Prf^{-/-}$) mice at the indicated E/T ratios for 2 h. Target NK cell death was analyzed by flow cytometry using propidium iodide (PI). Data are presented as the means plus SDs of triplicates. Representative data from two independent experiments are shown.