Supporting Information

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SI Experimental Procedures

Cell Culture and Reagents. Primary natural killer (NK) cells were obtained from peripheral blood of healthy donors in accordance with the Moffitt Cancer Center Institutional Review Board Human Subjects Board. NK cells were purified from peripheral blood mononuclear cells by negative selection (NK Isolation Kit; Miltenyi Biotech); purifications yielded >95% CD56+CD3− cells by FACS analysis. NK92 and primary NK cells were maintained in 20% (vol/vol) FBS AlphaMEM supplemented with 1% penicillin streptomycin (P/S), 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate (Gibco), MycoZap-PR reagent (Lonza), and 100 U/mL recombinant human IL-2 (Peprotech). K562, Raji, A549, and H1299 cell lines were maintained in 10% (vol/vol) FBS RPMI supplemented with 1% P/S, 1% L-glutamine (Gibco), and MycoZap-PR reagent (Lonza). HeLa and 293T cells were maintained in 10% (vol/vol) FBS DMEM supplemented with 1% P/S, 1% L-glutamine (Gibco), and MycoZap-PR reagent (Lonza). In all TGF-β stimulation experiments, purified platelet-derived human TGF-β1 (R&D Systems) was used at a concentration of 10 ng/mL.

Flow Cytometry and Immunoblot Analysis. Cells (1×10^6 cells per sample) were stained with the following antibodies: CD56-FITC (Southern), NKp44-PE (eBioscience), KIR2DS4-APC (Miltenyi), MICA/B (Santa Cruz, H-300), or HLA-A/B/C (BD Biosciences). For MICA/B and HLA-A/B/C staining, anti-rabbit Alexa488 (1:1,000) was added after the primary antibody. After washing, cells were resuspended in buffer containing 7-amino-actinomycin D (7-AAD) (Biolegend) and acquired on a FACS Caliber Flow Cytometer (BD Biosciences). Data were analyzed with FloJo Software (TreeStar). All plots gated on 7-AAD–negative cells. For immunoblot, NK cell lysates were acquired, and electrophoresis was conducted as described (1). Blots were probed with primary antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare). Primary antibodies included DNAX activating protein of 12 kDa (DAP12) (Santa Cruz; clone FL-113), KIR2DS4 (R&D Systems; clone 179315), NKp44 (R&D Systems; clone 253422), perforin (Ancell; clone delta G9), and β-actin (Invitrogen). Bands were visualized with ECL reagent (Amersham). Densitometry of protein signals was performed by using ImageJ software.

Chromium Release Assay. A ^{51}Cr-release assay was performed as described (1), by using Raji, A549, and H1299 tumor cells as targets. Targets were labeled with 200 μCi of Na [^{51}Cr] chromate (Amersham). Cells were washed and plated (5×10^3 cells per well) with NK cells. After 5 h incubation at 37 °C, supernatants were harvested and counted in a γ-counter. The percent specific ^{51}Cr release was calculated: [(experimental cpm − spontaneous cpm)/total cpm incorporated] × 100. All determinations were performed in triplicate, and the SEM of all assays calculated was typically ∼5% of the mean or less.

Quantitative RT-PCR. Cells were lysed in TRIzol (Invitrogen), and RNA was purified and converted to cDNA (iScript cDNA Synthesis kit; BioRad). Real-time PCR was performed with iScript SyberGreen Supermix (BioRad). Primer sequences are as follows: DAP12 (F 5′-GAGACCGAGTCGCCTTAT-3′/R 5′-ATACGGCCTCTGTGTGTTG-3′); GAPDH (F-5′-GAAGGTGAAGGTCGGAGT-3′/R-5′-GAAGATGGTGATGGGATTTC-3′); and Perforin (F-5′-ACTCACAGGCAGCCAACTTTGC-3′/R-5′-CTCTTGAAGTCAGGGTGCAGCG-3′). Experimental genes were normalized to GAPDH. PCR to detect mature microRNA (miR) transcripts was performed with Taqman reagents (Life Technologies). Purified RNA was reverse transcribed with primers for hsa-mir-183 (miR-183), hsa-mir-185 (miR-185), and RNU6B (RNU6B) followed by real-time PCR amplification with 6-carboxyfluorescein–conjugated primers specific for miR-183, -185, and RNU6B and No-Amp Erase Master Mix. Experimental miRs were normalized to RNU6B. Relative fold changes in expression were determined by using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).

miR Binding and BLAST Analysis. Analysis of the DAP12 3′ UTR (isoform 1, NM_003332) was performed by using publically available algorithms [miRANDA (microRNA.org), Targetscan (targetscan.org), and MirBase (mirbase.org)].

Cloning, Transfection, and Luciferase Assay. The oligonucleotide containing the DAP12 3′ UTR (synthesized by IDT) was amplified by RT-PCR (HotStar Mastermix; Qiagen) with the following primers: F, 5′-CTGATCAGTGGCGCTATGT-3′; R, 5′-GGGTTCGAACCCAGTACAAA-3′. Amplified products were run on a 1% agarose gel, extracted, (QIAquick Gel Extraction Kit, Qiagen) and subcloned into the pMIR-REPORT luciferase vector (Promega) to generate DAP12-Luc. The miR-183 seed-site mutant (DAP12-luc-M) was generated by Mutagenex. The constructs were verified for correct insert sequence and orientation by DNA sequencing. For luciferase reporter assays, HeLa cells were grown (70% confluent) in 12-well plates and transfected (Lipofectamine 2000; Invitrogen) with reporter constructs (500 ng per well), *renilla* luciferase (5 ng per well), and premiR-precursors (25 nm) (Applied Biosystems). At 24 h later, cells were lysed, and luciferase activity was quantified (Dual-Luciferase Reporter Assay; Promega) on a single automatic injection luminometer (Turner Biosystems). Ratios of *renilla* to firefly luciferase were quantified, and quadruplicates were averaged; experimental miRs were normalized to control miRs.

Argonaute 2–RNA Immunoprecipitation. Argonaute 2 (AGO2)–RNA immunoprecipitation was performed as described (2). Briefly, anti-AGO2 or rabbit IgG (Cell Signaling)-coated Sepharose beads (Santa Cruz) were created by incubating the beads overnight with either antibody at 4 °C. Beads were washed extensively and incubated with protein lysates from 24-h TGF-β–treated primary NK (1×10^7 cells per sample) or NK92 cells (5×10^6 cells per sample) for 24 h at 4 °C. Beads were washed extensively and incubated at 55 °C with proteinase K. Half of the pellet was washed and processed for quantitative PCR (qPCR); the other half was processed for immunoblot analysis. Membranes were probed with anti-rabbit–HRP (Sigma) for detection of AGO2 and equal protein input (Ig band).

NK Cell Lentiviral Infection. HIV-based lentiviral expression constructs (purchased from SBI) containing miR-183 (pCDH-CMV-MCS-EF1), antisense miR-183 (pGreenPuro shRNA), or scrambled control were packaged with third-generation packaging plasmids (PMD-g, PMD-Lg, Rev; a gift of Todd Fehniger, Washington University, St Louis) in 293T cells. Viral supernatants were harvested, filtered, and concentrated (Lenti-X Concentrator; Clontech). Primary NK cells (7.5×10^5 cells per well in 96-well flat-bottom plates) or NK92 cells (2.5×10^5 cells per well in 12-well plates) were centrifuged at $1{,}250 \times g$ with lentiviral particles (multiplicity of infection 20) in medium supplemented with 50 ng/mL recombinant human IL-15 (Peprotech). After two infections, cells were maintained in 20% (vol/vol) FBS AlphaMEM supplemented with 1% P/S, 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate (Gibco), MycoZap-PR reagent (Lonza), and 100 U/mL recombinant

human IL-2 (Peprotech). Infected cells were sorted (>95% GFP+) on FACS Aria (primary NK cells) or FACS Vantage (NK92 cells) cell sorters 7 d postinfection.

Immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) lung biopsies of 29 patients were obtained in accordance with the H. Lee Moffitt Cancer Center IRB Human Subjects Board. Tissues were sectioned and processed for immunostaining with the Vector ABC kit (Vector). Slides were deparaffinized with xylene, rehydrated in water/ethanol washes, subjected to antigen retrieval, blocked (Bloxall and Avidin/Biotin Blocking Kit), and stained with anti-human DAP12 (5 μg/mL) or TGF-β (LSBio; LS-B4772) (1 μg/mL) overnight at 4 °C. Next, a biotinylated antibody was added, followed by Vectastain ABC-AP reagent, development with VectorRed chromagen-tagged AP Substrate Kit 1, counterstaining with hematoxylin, and coverslipping with Vectashield mounting medium. DAP12-stained slides were scanned by using the Aperio ScanScope XT with a 200x/0.8-NA objective lens at a rate of 10 min per slide via Basler trilinear array. By using Spectrum software (Aperio), five 40,000-μm^2 fields from tumor and normal sections of each tissue (0.2 mm^2 per region per tissue) were selected by a study pathologist and extracted into the Definiens Tissue Studio software suite (Version 3.0). Cytoplasmic DAP12 protein staining was classified as high (+3), intermediate (+2), low (+1), or negative (0) categories based on the mean intensity of red chromagen associated with each nucleus. The sum of low, moderate, and high DAP12-expressing cells from five fields was determined for each sample; negative cells were excluded from the calculation.

Fluorescent Microscopy. NK92–Raji cell conjugates were analyzed for mobilization of perforin by immunofluorescence. TGF-β–treated or untreated NK92 cells were added to Cell-Tracker-Blue (Molecular Probes)-labeled Raji. Cells were incubated for 0 or 10 min at 37 °C, then cytospun, fixed with methanol/acetone (3:1), and stained with anti-perforin (1:200), followed by anti-mouse Ig-Texas Red (Sigma). Slides were then stained with anti-tubulin (1:3,500) and anti-mouse Alexa 488 (Invitrogen). Images of NK92–Raji conjugates were captured and evaluated in a blinded fashion. For quantification, images from 50 conjugates per treatment group were analyzed with Definiens Developer (Version 2.0; Definiens) as follows. First, Raji and NK92 cells were identified by using an autothreshold segmentation algorithm on the blue (Raji) and green (tubulin, NK92) fluorescence layers, respectively. Importantly, once they were identified as "blue" targets, Raji were not able to be identified as "green" despite staining positive for tubulin. Next, a 2-pixel-wide border was created where the target and NK cell made contact. Another autothreshold segmentation algorithm was used on the red fluorescence layer to identify perforin staining in the NK cells. Finally, the border and perforin demarcated regions were further segmented into individual pixels. The distance for each perforin pixel within a cell to the nearest border region pixel was extracted, and the average distance (in pixels) of the total perforin staining to border region was determined for each NK cell. The values were converted from pixels to micrometers with the following equation:

$$\text{Average Distance (pixels)} \times 0.1634\ \mu\text{m per pixel} = \text{distance } (\mu\text{m}).$$

For analysis of FFPE tissues by immunofluorescence, slides were processed as in *Immunohistochemistry*. Following blocking, anti-DAP12 (1.2 μL/mL) and anti-NKp46 (LSbio; LS-B2105 at 1:100) were added overnight at 4 °C, followed by anti-rabbit CF568 and anti-mouse CF647 (Biotium) (5ug/mL). Slides were mounted with Everbrite mounting medium (Biotium 23002) and viewed with a fully automated, upright Zeiss Axio- ImagerZ.1 microscope with a 40× 1.30-NA oil immersion objective, and DAPI, Texas Red, and FITC. Images were produced by using the AxioCam MRm CCD camera and Axiovision software suite (Version 4.6).

Tissue Microarray Construction/Immunostaining. Tissue cores (0.6 mm) from 150 adenocarcinoma (ADC) and 50 normal human lung tissues from Moffitt Cancer Center patients (obtained in accordance with the Moffitt Cancer Center IRB Human Subjects Board) were fixed in 10% (vol/vol) neutral buffered formalin for construction of a tissue microarray (TMA). Sections of the TMA (4 μm) were stained for dual NKp46 and DAP12 expression as described above. Slides were analyzed with an automated Zeiss Observer Z.1 inverted microscope through a 10×/0.3 NA and 20×/0.5NA objective with DAPI, Cy5, and Rhodamine filters. Images were captured by using the AxioCam MRm3 CCD camera and Axiovision (Version 4.7; Carl Zeiss). A total of 63 tumor and 19 normal cores were positive for NK cells (determined by NKp46+ stain). Th 10× images of NKp46+ cores were imported into Definiens Tissue Studio IF (Version 3.0) for analysis of DAP12 expression. NK cells were identified by Cy5 stain (NKp46) and by an average cell area of 151 μm; debris (area <10 μm) was excluded. Only DAP12 colocalized with NKp46 was analyzed. DAP12 colocalized with NKp46 was identified by the overlap of Rhodamine and Cy5 based on a constant threshold. Intensities of Cy5 and Rhodamine were independently quantified, and intensities for the stains of each cell were averaged for each tissue core. An H&E-stained serial section of the TMA was assessed by two board-certified pathologists and staged according to Tumor Node Metastasis (6th ed.) staging criteria. The number of NKp46+ ADCs falling in each stage was: stage IA, $n = 27$; stage IB, $n = 13$; stage IIA, $n = 4$; stage IIB, $n = 4$; stage IIIA, $n = 6$; stage IIIB, $n = 7$; stage IV, $n = 2$. There were 19 normal tissue cores containing NK cells.

Meta-Analysis of Published Lung Cancer Datasets and Data Analysis. *DAP12*, *TGF-β*, *MICA*, *MICB*, *ULBP1*, and *ULBP2* mRNA expression in human lung cancer and normal tissue was analyzed with microarrays cataloged in Oncomine (oncomine.com; Compendia Biosciences). All graphics are Oncomine.svg file output modified by Microsoft PowerPoint to enhance image quality. *P* values for all Oncomine data (Figs. 2*B* and 5*A* and Fig. S1*A*) were generated by Oncomine as described (3). All data are presented as means ± SEM or SD as indicated. For DAP12 expression in lung biopsies (Fig. 5 *C* and *D*), qPCR (Fig. 2*D*; Fig. 3 *A*, *C*, and *D*; and Fig. 4 *A* and *B*), receptor density (Fig. 1 *B* and *C*), and luciferase reporter assays (Fig. 3*B*), two-group comparisons were made by using paired Student *t* test with Microsoft Excel or GraphPad Prism. Significance was accepted at $P < 0.05$. For DAP12 and NKp46 expression analysis in the TMA among normal and tumor samples (Fig. 5 *G* and *H*), two-group comparisons were made by using unpaired Student *t* test with Welch's correction using GraphPad Prism.

1. Wei S, et al. (1998) Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: Identification of perforin and granzyme B mobilization by functional ERK2. *J Exp Med* 187(11):1753–1765.
2. Keene JD, Komisarow JM, Friedersdorf MB (2006) RIP-Chip: The isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nat Protoc* 1(1):302–307.
3. Rhodes DR, et al. (2004) ONCOMINE: A cancer microarray database and integrated data-mining platform. *Neoplasia* 6(1):1–6.

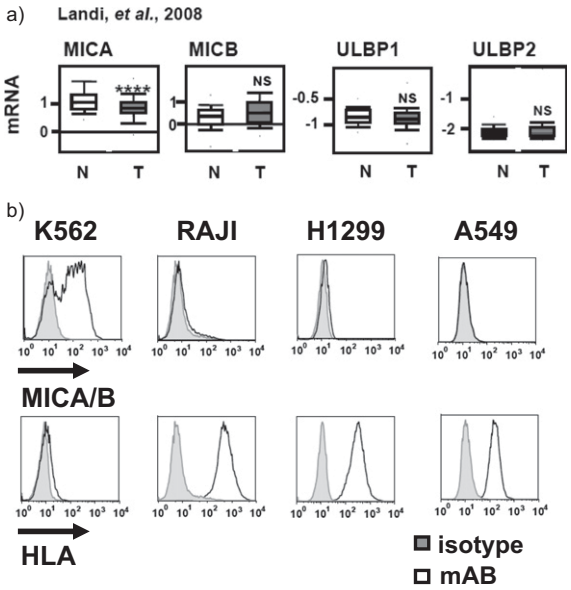

Fig. S1. (*A*) Meta-analysis of MICA, MICB, ULBP1, and ULBP2 mRNA expression in ADC and normal tissue from a gene expression microarray study published by Landi et al. (1) and cataloged in the Oncomine database (Compendia Bioscience). The *y* axis denotes the log2 median-centered probe signal intensity. Values from normal tissue (N; white boxes) and tumor tissue (T; gray boxes) are depicted. Error bars, SD. *P* values were generated by Student *t* test in Oncomine. (*B*) Analysis of NK ligands on target cells. K562, RAJI, A549, or H1299 tumor cells were stained with anti-MICA/B (top) or anti-HLA-A/B/C (bottom) and analyzed by flow cytometry with the MFIs depicted as histograms.

1. Landi MT, et al. (2008) Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. *PLoS ONE* 3(2):e1651.

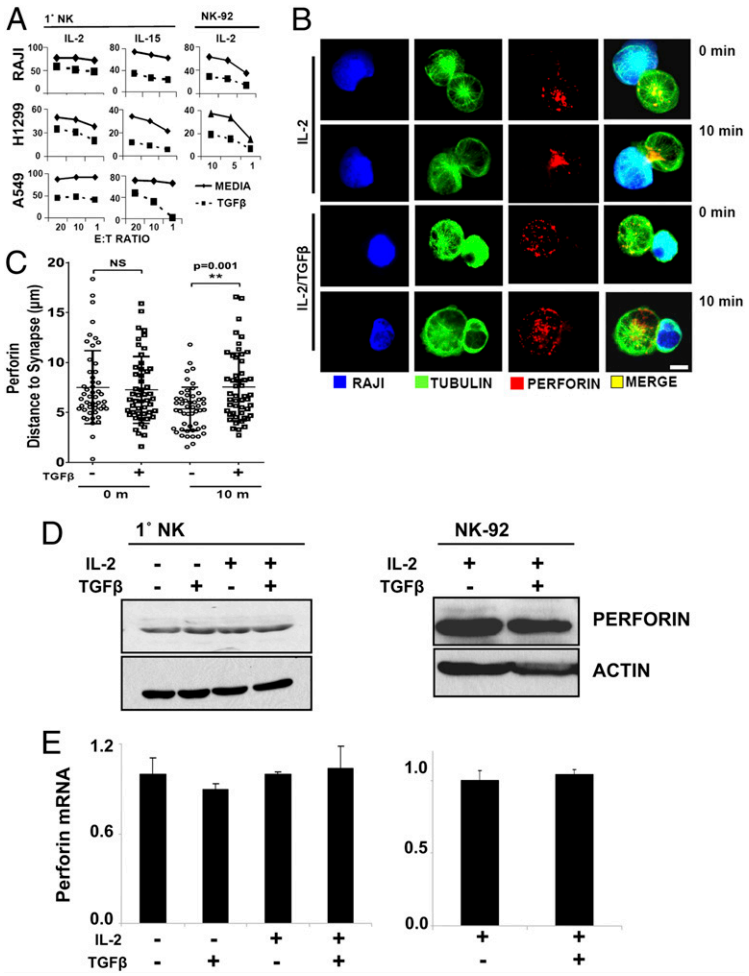

Fig. S2. (*A*–*C*) TGF-β alters NK cell phenotype and cytotoxic potential. (*A*) Effect of TGF-β on NK cytolytic function. Primary NK or NK92 cells cultured 72 h with IL-2 (100 U/mL), IL-2/TGF-β (10 ng/mL), IL-15 (10 ng/mL), or IL-15/TGF-β were plated with ^{51}Cr-labeled Raji, A549, or H1299 target cells. Cytotoxicity was determined by ^{51}Cr release into supernatants. (*B*) Effect of TGF-β on immune synapse formation. NK92 cells were treated with TGF-β (72 h) and cultured with Celltracker Blue-labeled Raji tumor cells for 0 or 10 min. Cells were fixed, cytospun, and stained with anti-perforin and -tubulin. Representative conjugates are pictured at 400× magnification. (Scale bar, 10 μm.) Results are representative of three experiments. (*C*) Quantification of perforin movement. The distance of each perforin granule to the conjugated tumor cell was measured and averaged over the total perforin granules per cell ($n = 50$ conjugates per group). (*D* and *E*) Assessment of perforin in TGF-β–treated NK cells. (*D, Left*) Freshly isolated primary NK were cultured in medium, medium/TGF-β, medium/IL-2, or medium/IL-2/TGF-β. (*Right*) NK92 were cultured in medium with IL-2 or IL-2/TGF-β. After 72 h, cells were lysed for immunoblot analysis of perforin; β-actin served as loading control. (*E*) qPCR analysis of perforin (normalized to GAPDH) in primary NK or NK92 cells treated as in *D* for 48 h. Error bars, SEM.

a **DNAX activating protein, 12 kDa (DAP12)**
Gene: TYRO protein tyrosine kinase binding protein (*TYROBP;NM_003332, NM_198125*)
TGA-1-
GCC CGA AUC AUG ACA GUC AGC AAC AUG AUA CCU GGA UCC AGC CAU UCC UGA AGC CCA CCC UGC ACC UCA UUC CAA CUC CUA CCG CGA UAC AGA CCC ACA G**AG UGC CAU** CCC UGA GAG ACC AGA CCG CUC CCC AAU A**CU CUC C**UA AAA UAA ACA UGA AGC AC-161

DNAX activating protein, 10 kDa (DAP10)
Gene: hematopoietic cell signal transducer (*HCST*; NM_001007469, NM_014266)
TGA-1-
CCC UCC UGC AGC UUG GAC CUU UGA CUU CUG ACC CUC UCA UCC UGG AUG GUG UGU GGU GGC ACA GGA ACC CCC GCC CCA ACU UUU GGA UUG UAA UAA AAC AAU UGA AAC ACC-111

b

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     3' ucacuuaagauggUCACGGUAu 5' hsa-miR-183
                       ||||||||
 88:5' uacagacccacagAGUGCCAUc 3' TYROBP
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     3' aguccuugacggaaaGAGAGGu 5' hsa-miR-185
                        ||||||
122:5' gaccgcucccccaauaCUCUCCu 3' TYROBP
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Fig. S3. miR-183 targets the human DAP12 3′ UTR. (*A*) Nucleotide sequence of the human DAP12 (*TYROBP*) and DAP10 (*HCST*) 3′ UTRs. Sequences begin with the translation termination codon (TGA) and begin with nucleotide 1 of the UTR; the number following each sequence tells its nucleotide length. miR-183 and -185 sites are in bold and underlined in the DAP12 3′ UTR; these sequences are absent in the DAP10 3′ UTR. (*B*) Alignment of human DAP12 (TYROBP) 3′ UTR mRNA and miR-183 or -185 as depicted by the miRanda algorithm (microRNA.org). The number labeling the 5′ end of TYROBP mRNA represents the nucleotide position downstream of the translation termination codon. Capital letters constitute the miR seed sequences; vertical lines depict miR binding to mRNA.

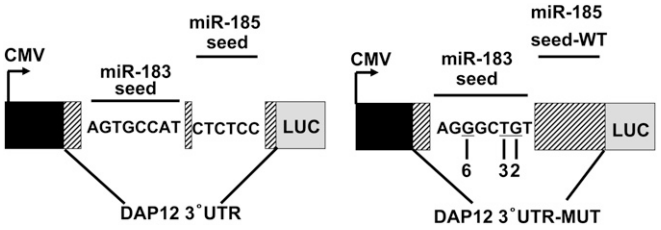

Fig. S4. Schematic of luciferase reporter constructs carrying wild-type (DAP12-luc) or mutated (DAP12-luc-MUT) DAP12 3′ UTR. The miR seed sequences are depicted as Arabic letters; nucleotides mutated in DAP12-luc-MUT are underlined. Vertical lines and numbers in the mutated sequence depict the seed positions altered within the 3′ UTR. CMV, cytomeglavirus promoter; LUC, luciferase.

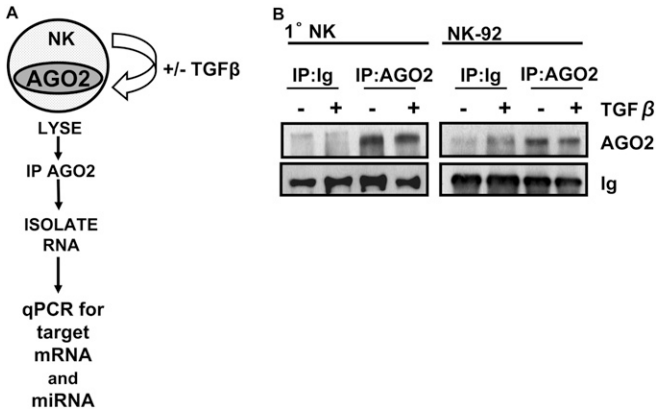

Fig. S5. AGO2–RNA immunoprecipitation. (*A*) Experimental schematic. Freshly isolated primary NK or NK-92 cells were treated with IL-2 or IL-2/TGF-β for 24 h followed by lysis for protein extraction. Protein lysates were applied to AGO2-or isotype-coated beads after which RNA was extracted and processed for qPCR or immunoblot analysis. (*B*) Immunoblot of immunoprecipitated complexes. Membranes were probed with anti-rabbit IgG-HRP to detect rabbit anti-human AGO2 used for immunoprecipitation; Ig band demonstrates equal input of cellular lysates.

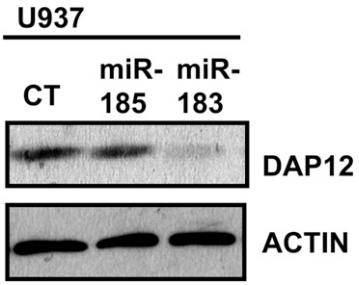

Fig. S6. miR-183 is sufficient to deplete DAP12 in myeloid cells. U937 myeloid cells (constitutively DAP12+) were transfected with scrambled control (CT), miR-185, or -183 precursor oligonucleotides. At 48 h later, cells were lysed for immunoblot analysis; β-actin serves as a loading control.

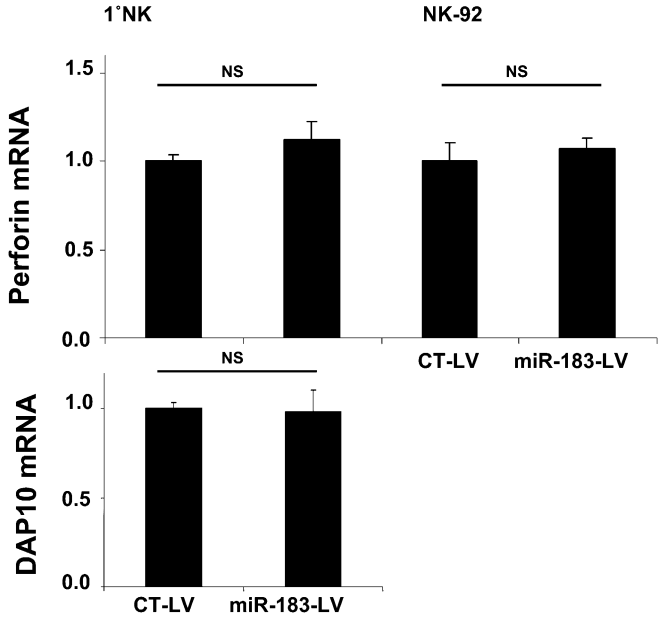

Fig. S7. miR-183 overexpression in NK cells does not affect perforin or DAP10 expression. Lentiviral expression constructs containing scrambled control (CT-LV) or miR-183 (miR-183-LV) were packaged into viral particles for NK cell infection. Infected (GFP+) primary NK or NK92 cells were sorted to >95% purity, followed by qPCR analysis of perforin (*Upper*) or DAP10 (*Lower*) (normalized to GAPDH). DAP10 mRNA was not detected in NK92 cells and is not depicted. Error bars, SEM; NS, not significant.

Table S1. Histology and grading of lung cancer biopsies

Histological classification	Tumor grade
Normal	NA
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	1
Adenocarcinoma	2
Adenocarcinoma	3
Broncioalveolar carcinoma	1
Broncioalveolar carcinoma	1
Broncioalveolar carcinoma	1
Broncioalveolar carcinoma	1
Broncioalveolar carcinoma	1
Large cell carcinoma	3
Large cell carcinoma	3
Neuroendocrine carcinoma	1
Neuroendocrine carcinoma, large cell type	2
Squamous cell carcinoma	1
Squamous cell carcinoma	1
Squamous cell carcinoma	2
Squamous cell carcinoma	2
Squamous cell carcinoma	2
Squamous cell carcinoma	2
Squamous cell carcinoma	3
Squamous cell carcinoma	3
Squamous cell carcinoma	3
Squamous cell carcinoma	3
Squamous cell carcinoma	3

Histological characterization and tumor grades of FFPE tissues from Moffitt Cancer Center lung cancer patients (n = 29). Biopsies from 29 patients with suspected lung malignancies were resected; each specimen contained suspected malignant regions as well as adjacent normal tissue, as determined pathological designation. The histological characterization and tumor grading shown was conducted by two board-certified pathologists. 1, well-differentiated; 2, moderately differentiated; 3, poorly differentiated. NA, not applicable.