Online Supplemental Material

Monoclonal Antibodies, immunofluorescence procedures and flow cytometry. Cell suspensions from the human tumors and lymphocyte samples were prepared and, where appropriate, Fc receptors were blocked using human serum. Antibodies to the following antigens were used for staining human melanoma cell lines: HLA-A, -B and -C (clone: W6/32HL), HLA-B-C (B1.23.2), ICAM-1 (VF 27-516.1), MICA (BAM 195 (1)), MICA/B (6D4), ULBP1 (M295), ULBP2 (M310), ULBP3 (M550) and ULBP4 (M478) (ULBP-specific antibodies were a kind gift of D. Cosman, Amgen, Seattle, WA), CD155 (L95), CD112 (L14) (L95 and L14 were characterized as described in Ref. 15 from the main manuscript), NKG2D (1D11) (Becton Dickinson). R-PE- and FITC-conjugated goat anti-mouse IgG antibodies were purchased from Jackson Immuno Research Laboratories (Baltimore, MD, USA) and Sigma, respectively. R-PE-conjugated F (ab')$_2$ fragments of goat anti-human Fc antibodies were from Jackson Immuno Research Laboratories. To define melanoma cells, mouse mAbs specific for HMB-45 (Dako Cytomation, Hamburg, Germany) and S100 (Dianova, Hamburg, Germany) were used. CD3-specific mAb (OKT3) was obtained from ATCC (Manassas, VA) while CD56-specific mAb (NCAM-16) was purchased from Becton-Dickinson. Mouse cell suspensions were prepared and, where appropriate, Fc receptors were blocked with anti-CD16 (clone 2.4G2). Antibodies and fusion proteins to the following molecules were used to stain murine melanoma tumors and lymphocytes: CD112 (TX78 or TX75), CD155 (TX56), CD226 (TX42) (2); H-2D^b (KH95), H-2K^b (AF6- 88.5), CD48 (HM48-1), CD54 (3E2), NK1.1 (PK136), CD3 (145-2C11) (BD Biosciences); NKG2D (CX5) (eBioscience); mouse NKp46-Fc and mouse NKG2D-Fc (R&D Systems); anti-rat IgG (Sigma-Aldrich); anti-rat IgG2a (MRG2a-83) (Biolegend); F(ab)'$_2$ goat anti-human Fc (Jackson ImmunoResearch).

Appropriate isotype controls were used to estimate background fluorescence. Acquisition was performed using a FACSCalibur or LSRII (BD Biosciences) with dead cells excluded based on scatter profile or DAPI inclusion. Analysis was performed using FlowJo (Tree Star) and Cell Quest softwares. In receptor blocking experiments IgM mAb to the following receptors were used: NKp46 (KL247), NKp44 (KS38) (both kindly provided by S. Parolini, University of Brescia, Brescia, Italy) and NKp30 (F252) (3) and IgG to the following receptors were used: NKG2D (BAT221) and DNAM-1 (F5). Control Abs in receptor blocking experiments were T345 (IgG, which recognizes a lymphocyte membrane-associated vaccinia virus growth factor) and TIB200 (IgM, anti-CD57, ATCC).

Human NCR-Fc-chimeric constructs, transfections, protein expression and purification. To study the surface expression of NCR ligands by cytofluorimetric analysis, the cDNA sequences coding for the extracellular domain of NCR1 (4), NCR2 (5) and NCR3 (3) have been subcloned in frame to the sequence encoding human IgG1-Fc domain. More precisely, to decrease the FcR-binding, soluble molecules have been produced containing mutation in three amino acids located in the hinge region (Leu234Ala, Leu235Glu and Gly237Ala) using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and a pair of oligodeoxynucleotide primers complementary to each other and containing the three specific mutations (5'-AGG CAC CTG AAG CCG AGG GGG CAC CGT CAG TCT T-3'; and 5'-AGG ACT GAC GGT GCC CCC TCG GCT TCA GGT GCC T-3'). These residues have been described to be critical for determining IgG receptor affinity implicated in specific bindings (6). The cDNA sequences coding for NCR1 (from amino acid 1-255), NCR2 (amino acids 1-190) and NCR3 (residues 1-138) have been obtained by PCR and cloned

in the pRB1-IgG1-FLAG vector (R. Biassoni, unpublished) containing the cDNA coding the human IgG1-Fc region modified from the CD5neg vector (7). Stable transfectants have been generated using HEK293 human cell line (human embryonic fibroblast) and soluble-Fc proteins have been produced adapting cells to grow in SFM II synthetic medium (Invitrogen Corporation, Carlsbad, CA, USA). The soluble molecules have been purified by affinity chromatography utilizing Protein A Sepharose 4 Fast Flow (GE Healthcare Amersham Pharmacia Biotech; Piscataway, NJ, USA) and checked for purity by SDS-PAGE followed by silver staining.

Clonal and polyclonal NK cell generation. Clonal human NK cell preparation was done as described previously (8)**.** For polyclonal NK cell preparation, PBMCs were isolated by Ficoll-paque (Biopaque, Berlin, Germany) density gradient centrifugation and subsequently enriched for NK cells using the NK cell Isolation kit and VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the isolated $CD3^{-}CD56^{+}$ NK cell populations was above 95%. This protocol was also applied to isolate NK cells from frozen PBMCs of cancer patients. Freshly-enriched NK cells were suspended in IMDM culture medium (Life Technology, Milan, Italy) supplemented with Penicillin (100 IU/ml) and Streptomycin (100 μg/ml) and 10% FBS. Activated polyclonal NK cells, were obtained by culturing enriched NK cells in IMDM medium supplemented with rIL-2 (1000 U/ml) for two days. Polyclonal NK cells used in receptor blockade experiments were activated with 250 U/ml rIL-2 for 3-4 days before use. For mouse NK cells, splenocytes were enriched for NK cells with the mouse NK Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's directions. For IL-2-activated killer cells, enriched cells were cultured at $1x10^6$ cells/ml in RPMI/10% FCS/2mM L-glutamine/50_M 2-mercaptoethanol supplemented with 1000

U/ml recombinant human IL-2 (Chiron). For *in vivo* activated NK cells, mice were given 100 _g polyI:C (Sigma-Aldrich) i.p. and splenocytes were harvested 18-24 hours later.

NK cell cytotoxicity assay. The fluorescent c'FDA NK assay and the standard ^{51}Cr release test were performed using the protocol described elsewhere (9). The results of some experiments (Fig 4) were validated using ^{51}Cr release assays. Briefly, target cells were collected, washed with complete media and labeled with Na2 $^{51}CrO4$ (0.1 _Ci/106 cells) for 45-60 minutes at 37^0C. The cells were washed twice with complete media before being added in triplicate to effector cells at the indicated E:T ratios. Co-cultures were incubated for 4 hours at 37^0C before the supernatants were sampled and the chromium release was determined by a scintillation counter. In some assays, receptor-ligand interactions were blocked by pre-incubation of the NK cells or target cells with the indicated blocking reagent before co-culture. The percent specific lysis was calculated as (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. All antibodies were used at a final concentration of 10_g/ml.

Double Determinant Assay. To analyze the MICA/B released by the melanoma metastases, cell culture supernatants were diluted twice and added to EIA/RIA plates. The plates were processed as described elsewhere (10).

Immunohistochemistry. Acetone fixed tumor tissue sections were incubated with antibodies directed against melanoma markers (polyclonal antisera for HMB-45 and S100), HLA class I, Nectin-2, PVR, MICA/B, CD3, and CD56. After washing, sections were incubated with biotinylated species-specific secondary antibodies. Binding of biotinylated antibodies was detected either by adding streptavidin-horseradish peroxidase (Jackson Immunoresearch, Dianova) and AEC substrate (Sigma, Taufkirchen, Germany)

or by using the Vestastain ABC-AP Kit (Vector Laboratories, Linaris, Wertheim, Germany) and Vector Red substrate (Vector Laboratories).

References

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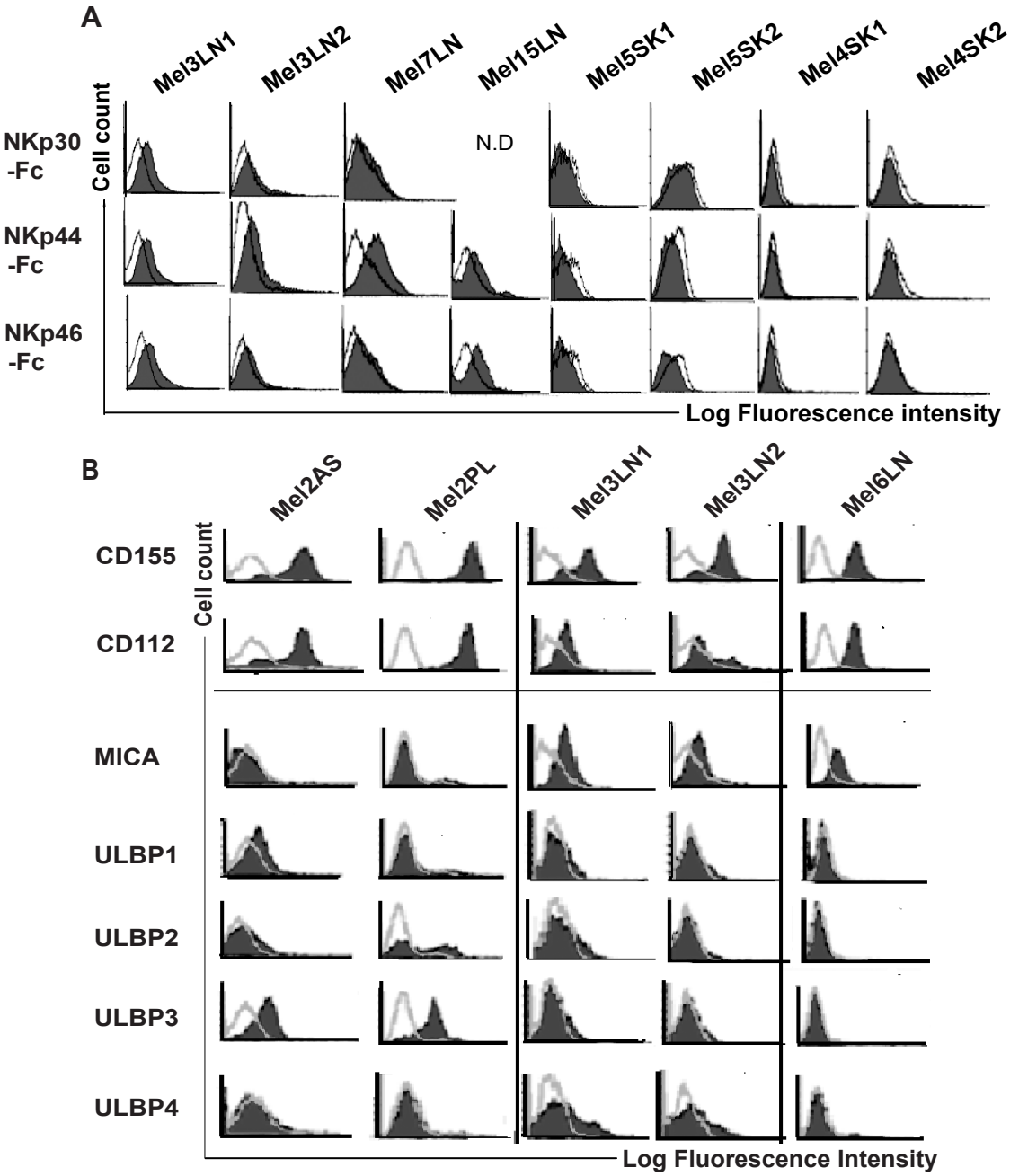
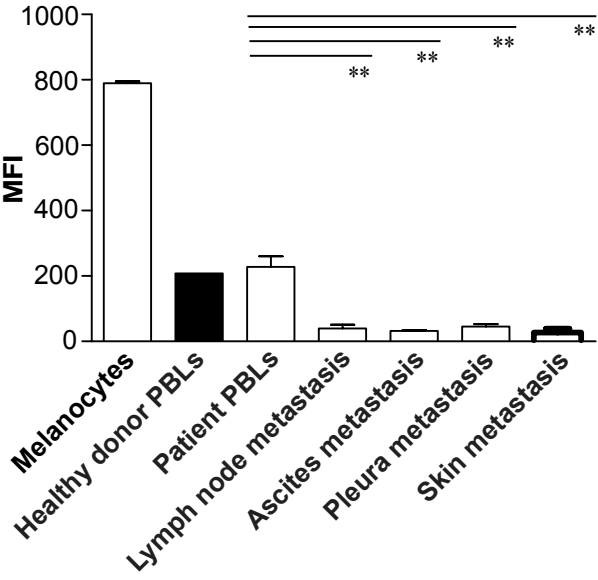
Supplementary Figure 1



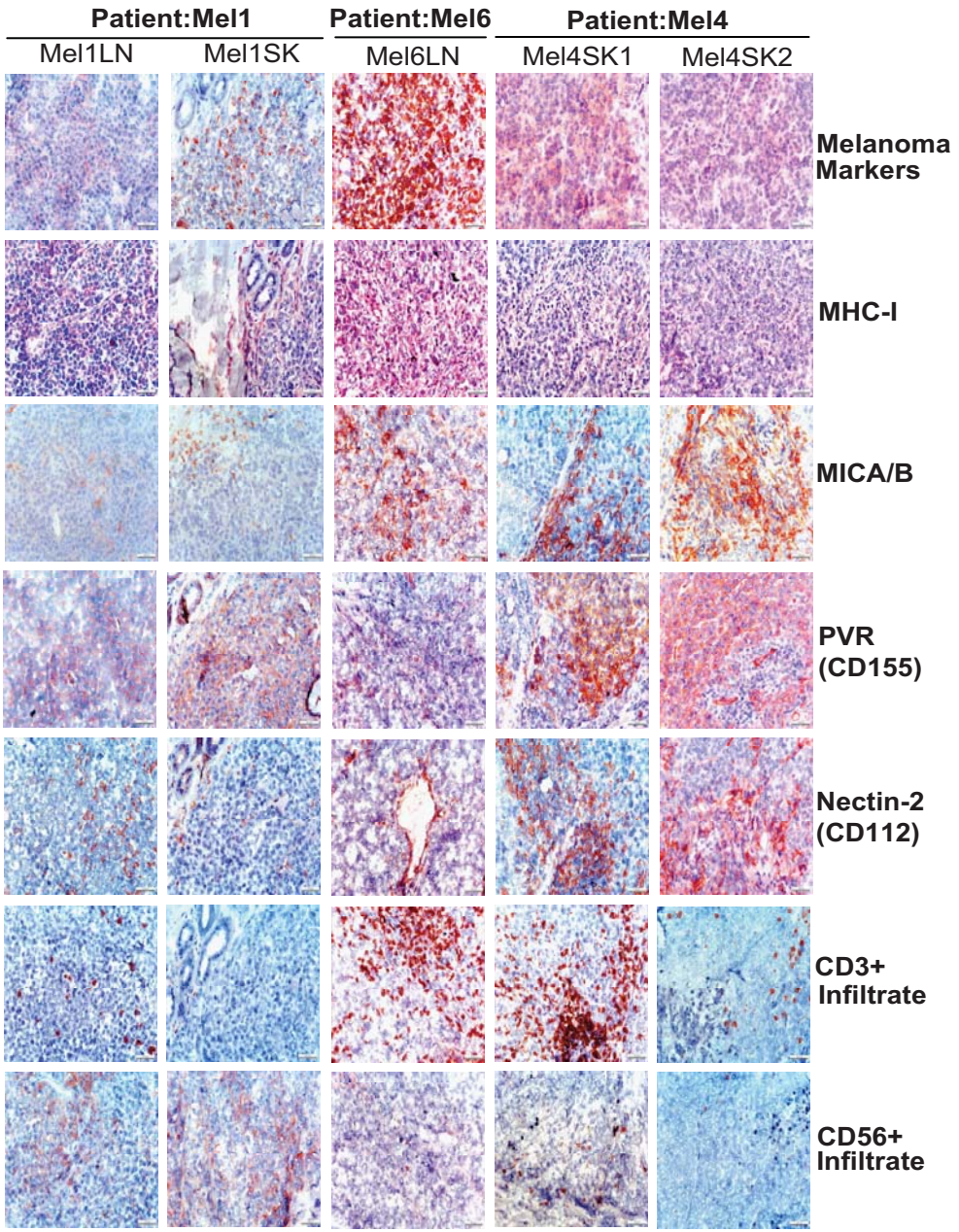
Supplementary Figure 1. Expression of ligands for NCRs and DNAM-1 on human melanoma metastatic cells. (A) NCR ligand expression was measured by flow cytometry on unpaired metastases obtained from lymph node (LN), ascites (AS), pleura (PL) and skin (SK) of different patients. Staining with NKp30-Fc, NKp44-Fc and NKp46-Fc (filled profiles) was compared to staining with irrelevant CD5-Fc (open profiles); **(B)** DNAM-1 and NKG2D ligand expression (filled profiles) measured by flow cytometry on melanoma cells obtained from different anatomical sites of patients (as indicated in the figure). Negative controls were stained with FITC-conjugated anti-mouse Ig xenoantibodies (open profiles). Histograms are representative of 2-5 independent experiments.

Supplementary Figure 2



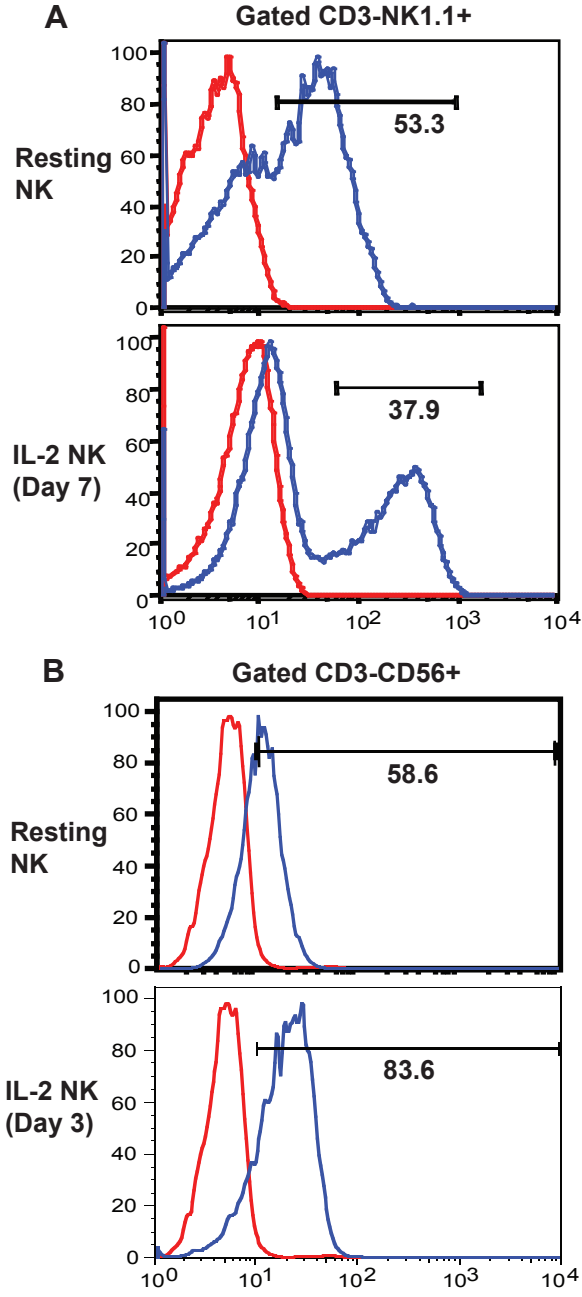
Supplementary Figure 2. HLA class I downregulation of human melanoma metastatic cells obtained from different anatomical sites. The lymph node metastases Mel3LN1, Mel3LN2 and Mel9LN; the ascites metastatic cell line Mel2AS; the pleura metastatic cell line Mel2PL and the skin cell line Mel5SK1 were stained with HLA class I antigen-specific mAb W6/32 and FITC-conjugated anti-mouse Ig xenoantibodies. Stained cells were analyzed by flow cytometry. Commercially available melanocytes (Promocell), Peripheral blood lymphocytes (PBLs) isolated from patients Mel2, 3, 5 and 9 and from 4 healthy donors were used as positive controls. Data are mean$\pm$S.D. of 3 experiments performed and the statistical significance of the differences was calculated utilizing the ANOVA analysis. ** $P < 0.005$ compared between PBLs and corresponding melanoma metastases.

Supplementary Figure 3



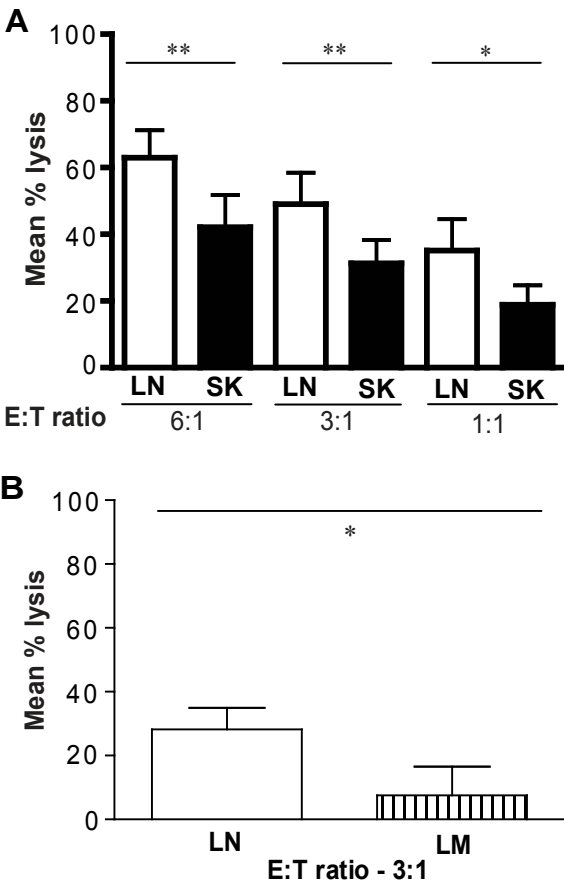
Supplementary Figure 3. Immunohistochemistry on biopsies of human melanoma metastases. Tissue sections from frozen tumor metastases were acetone fixed and stained with mAb specific for the following molecules: HMB-45 or S100 (both melanoma markers), HLA class I (clone W6/32), MICA/B (6D4), Nectin-2 (L14), PVR (L95), CD3 (OKT3) and CD56 (NCAM-16). The histology was performed on serial tissue sections obtained from patients Mel1, 4 and 6. All the analysis was performed on overlapping tissue sections except for Mel1LN where the staining for HLA class I and CD56 infiltrate was done on another section from the same tumor tissue. Ab staining is shown in red and hematoxylin counterstain is in blue. All images are in original magnification, x100.

Supplementary Figure 4



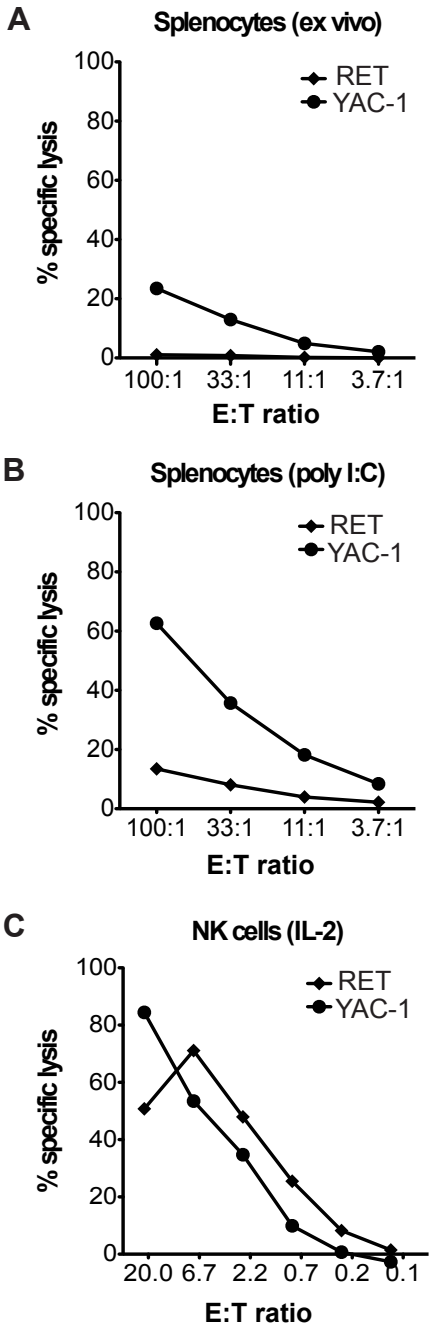
Supplementary Figure 4. Expression of DNAM-1 on murine and human NK cells. (A) The expression of DNAM-1 receptor on CD3-NK1.1+ NK cells was determined directly on resting cells ex vivo, or following 7 days culture with IL-2. **(B)** The DNAM-1 expression on CD3-CD56+ NK cells was determined directly on resting cells ex vivo, or following 2 days culture with IL-2. Numbers on markers indicate % of DNAM-1+ NK cells, using isotype controls (red) to determine the background. Analyses is representative of 2-4 experiments.

Supplementary Figure 5



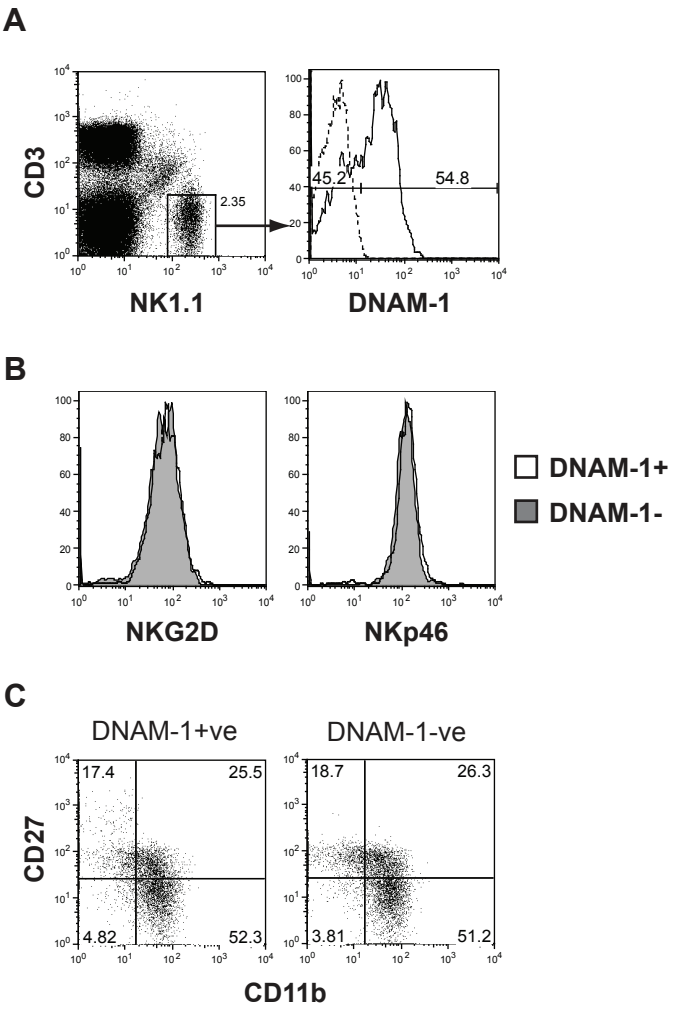
Supplementary Figure 5. Statistical analysis of LN and other metastases susceptibilities to NK cell lysis. (A) ANOVA and post hoc analysis (Fischer's exact test) of the differences between lymph node (LN) and skin (SK) metastatic cell lines using resting NK cells and activated NK cells (data not shown). The LN cell lines used were Mel3LN1, Mel3LN2, Mel6LN, Mel7LN, Mel12LN, Mel13LN and Mel14LN; the SK cell lines were Mel1SK, Mel4SK1, Mel5SK2, Mel8SK1, Mel8SK2 and Mel14SK. Data are mean±SEM of % lysis obtained from 4 independent experiments. ** $P < 0.005$ at E:T ratios 6:1 and 3:1, * $P < 0.05$ at 1:1. **(B)** Student's t-test analysis of % lysis between lymph node (LN) and uveal melanoma liver metastatic liver metastatic (LM) cell lines susceptibilities to resting NK cells. The LN cell lines were Mel3LN1 and Mel1LN; the LM cells were OMM1 and OMM2.3. Data are mean±SEM obtained from 3 independent experiments. * $P < 0.05$.

Supplementary Figure 6



Supplementary Figure 6. Activated NK cells kill RET melanoma cells. Cytotoxicity of fresh, in vivo activated and IL-2 cultured NK cells versus YAC-1 and RET cells is shown in A, B and C respectively. The E:T ratio and the % specific lysis is shown in x-and y-axis respectively.

Supplementary Figure 7



Supplementary Figure 7. Phenotypic analysis of DNAM-1+ and DNAM-1- NK cell subsets. (A) Gating strategy for the identification DNAM-1+ and DNAM-1- NK cell subsets. NK1.1+CD3- cells were gated as shown (dot plot) and DNAM-1+ and DNAM-1- subsets (histogram) were determined by labeling with TX42 anti-DNAM-1 mAb (solid line). Isotype control antibody was used to determine background signal (dashed line). Numbers in plots represent percentage of cells in lymphocyte gate (dot plot) and NK cell gate (histogram). **(B)** Expression of NKG2D and NKp46 receptors on DNAM-1+ (open histogram) and DNAM-1- (shaded histogram) NK cells. NK cells were gated as in (A). **(C)** Maturation of DNAM-1+ and DNAM-1- NK cells according to expression of CD27 and CD11b. NK cells were gated as in (A). Numbers in quadrants are the percentage of the DNAM-1 subset. Results are representative of at least 2 experiments.

Supplementary Table 1

	Human (lines)		Mouse (Primary)	Mouse (Cell lines)
	LN	Others		
NCR LIGANDS				
NKp30	–	–	NA	NA
NKp44	++	+/-	NA	NA
NKp46	++	+/-	++	++
ADHESION MOLECULES				
CD155	++	++	++	++
CD112	+	+	+/-	+
ICAM-1	++	++	–	–
CD48	ND	ND	–	–
NKG2D LIGANDS				
MICA	+/-	+/-	NA	NA
MICB	+/-	+/-	NA	NA
ULBP1	–	–	NA	NA
ULBP2	–	–	NA	NA
ULBP3	+/-	+/-	NA	NA
ULBP4	–	–	NA	NA
Rae-1	NA	NA	–	–
MULT-1	NA	NA	–	–
H60	NA	NA	–	–
MHC CLASS I MOLECULES				
HLA - I	–	–	NA	NA
H-2K	NA	NA	–	–
H-2D	NA	NA	–	–
COSTIMULATORY MOLECULES				
CD40	ND	ND	–	–
CD80	ND	ND	–	–
CD86	ND	ND	–	–

ND = Not Done; NA = Not Applicable, due to species-specific differences.

Supplementary Table 1. Comparative summary of ligand expression in human and mouse melanoma.