Tumor-Primed Human Natural Killer Cells Lyse NK-Resistant Tumor Targets: Evidence of a Two-Stage Process in Resting NK Cell Activation

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Tumor-Primed Human Natural Killer Cells Lyse NK-Resistant Tumor Targets: Evidence of a Two-Stage Process in Resting NK Cell Activation

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NK cells are defined as those cells that lyse tumor cells without priming. In this study, we show that the preincubation of resting human NK cells with the leukemia cell CT1-1 primes NK cells to lyse NK-resistant cell lines, primary leukemias, and solid tumors even when HLA-matched, allogeneic or autologous. The primed NK cells remained nonresponsive to HLA-C matched and mismatched normal mononuclear cells from multiple donors. CD69, a known NK trigger receptor, was shown to be the predominant trigger on the tumor-primed NK cells because lysis was blocked with the rCD69 protein. The lack of lytic activity against normal hematopoietic cells implied that the ligand for CD69 is tumor restricted, and this was confirmed by experiments using fluorochrome labeled rCD69. It has been recently shown that resting NK cells require prior stimulation with IL-2 before triggering by all known NK-triggering ligands. In this study, we show that a tumor cell can provide the NK priming signal independently of IL-2. These data provide evidence for two NK evasion strategies for tumor cells, namely the prevention of priming (type 1 evasion) and failure to trigger (type 2 evasion). Most NK-resistant cell lines are type 1 and fail to prime resting NK cells but are lysed by IL-2-primed NK cells. In contrast, CT1-1 cells prime resting NK cells but fail to trigger (type 2), and coincubation with CT1-1 primes for triggering by type 1 NK-resistant tumor cells. These tumor-activated NK cells lyse a broad spectrum of tumor cells with a degree of specificity never previously reported. The Journal of Immunology, 2007, 178: 85–94.

Human NK cells mediate the lysis of tumor cells and virus-infected cells via natural cytotoxicity and Ab-dependent cellular cytotoxicity and are controlled by positive and negative cytolytic signals (1, 2). Negative (inhibitory) signals are transduced by some killer Ig-like receptors (KIRs), by ILT2/LIR, and by the C-lectin domain-containing receptor CD94/ NKG2A (2). The regulation of NK lysis by inhibitory signals is known as the “missing self” hypothesis in which specific HLA-class I alleles expressed on the target cell surface ligate inhibitory receptors on NK cells. The down-regulation of HLA molecules on some tumor cells and some virally infected cells (e.g., CMV) is believed to lower this inhibition below a target threshold, making the target cell susceptible to NK cell-mediated lysis. Inhibitory receptors fall into two groups, those of an Ig-superfamily, the KIRs and those of the lectin family, the NKG2s, which form dimers with CD94 at the cell surface. KIRs have a two- or three-domain extracellular structure and bind to HLA-A, -B, or -C. The NKG2/CD94 complexes ligate HLA-E.

Inhibitory KIRs have up to four intracellular domains that contain ITIMs and the best characterized are KIR2DL1 (CD158a), KIR2DL2, and KIR2DL3 (CD158b), which are known to bind HLA-C molecules. KIR2DL2 and KIR2DL3 bind the group 1 HLA-C alleles, while KIR2DL1 binds to group 2 alleles. KIR3DL1 (CD158c1) binds to HLA-Bw4 motifs.

Transfection of appropriate HLA-C alleles into NK-susceptible target cells can render them resistant to NK lysis (3), and tumor cell lines such as RAJI are NK resistant through constitutive expression of type 1 and type 2 HLA-C alleles. However, resistance to NK-mediated lysis is overcome by preincubation of NK cells with IL-2 and the generation of “lymphokine-activated killer cells”.

Activated NK cells are capable of multiple cell lysis, and it is possible that activation through interaction with a susceptible target cell provides sufficient stimulus to overcome HLA-mediated inhibition in a manner analogous to that mediated by IL-2. Although this would be an efficient mechanism for expanding antitumor cytolytic in vivo, it could lead to autoreactivity to normal cells. We hypothesized that NK cell-mediated lysis may require both priming and triggering. The priming signal may be nonspecific, such as IL-2 or IFN-γ, or provided by a tumor cell. The triggering signal should be specific to prevent autoreactivity. A potential candidate for a tumor-restricted initiation signal is the tyrosine kinase domain epitope of heat shock protein 70 as recently described (4).

The triggering receptors responsible for natural cytotoxicity (NCRs) remain largely elusive but include CD2, CD38, CD69, NKR-P1, NKp46, NKp30, and NKp44. Recently, it has been elegantly demonstrated that ligation of these molecules is inadequate to trigger lysis by resting NK cells, which need some form of prior
stimulation such as nonspecific activation with IL-2 (5). This finding argues that freshly isolated resting NK cells need a two-stage process of activation and triggering for natural cytotoxicity, in contrast to Ab-dependent cellular cytotoxicity where CD16 ligation is capable of the direct triggering of lysis by resting NK cells (5).

We have been interested in the role of CD69 as an NCR following the observation that it initiates NK lytic activity in a reverse cytotoxicity assay (6) and that it caps at the synapse between NK cells and autologous acute myeloid leukemia (AML) blasts (7), leading to lysis of the leukemic cells. CD69 is a homodimeric C-type lectin (8). Its physiological ligand is unknown, as is the tissue distribution of the ligand.

To test the two-step hypothesis of NK activation and lysis, we established a test system in which an NK-resistant acute lymphoid leukemia cell line (CTV-1) was used to stimulate NK cells for up to 24 h in vitro, and the resultant activated NK cells were tested for the ability to lyse NK-resistant tumor cells and normal peripheral blood and bone marrow mononuclear cells.

Materials and Methods

Cell culture reagents

All cell cultures were maintained in complete media consisting of RPMI 1640 supplemented with 10% FCS and penicillin (100 IU) and streptomycin (100 IU) (all supplied by Invitrogen Life Technologies).

Immunophenotyping

To analyze cell surface Ag expression, 10^5 cells in 100 μl of HBSS were incubated with fluorochrome-conjugated mAbs at the manufacturer’s recommended concentration (BD Biosciences) for 15 min at room temperature. After washing, the cells were analyzed by flow cytometry (FACS-Calibur with CellQuest software; BD Biosciences). Forward and side light scatter characteristics were used to gate on the viable lymphocyte population before acquisition of at least 10,000 cells from each sample. All fluorochrome-conjugated mAbs were purchased from BD Immunocytometry Systems or Beckman Coulter.

Isolation of human NK cells and target cells

All samples were obtained with informed consent for research into innate immunity to leukemia or for a study of immunity to breast cancer and ovarian cancer. Fresh heparinized peripheral blood samples were obtained from normal healthy donors and from patients with acute and chronic leukemias at diagnosis (Table I). Additionally, samples were obtained from two HLA-identical sibling donors of patients selected for allogeneic stem cell transplant and from five patients in clinical remission from AML. The patients had donated bone marrow samples at the time of their disease presentation and the leukemic blasts were cryopreserved in multiple aliquots.

PBMCs were isolated from venous blood by discontinuous density gradient separation (Lymphoprep) and typed for HLA class I A and B alleles by low resolution techniques and for HLA-Cw to high resolution. CD56^CD3^- cells were purified from PBMCs by direct immunomagnetic separation with a CD56 MultiSort kit (Miltenyi Biotec) and subsequent depletion with CD3 FITC and anti-FITC beads. All selected cells were confirmed as $>$98% CD56^ and $<$3% CD3^ and resuspended in complete medium. In some experiments NK cells were subsequently labeled with mAbs specific for CD158a (KIR2DL1) (clone HP-3E4), CD158b (KIR2DL2/3) (clone GL183), and CD158e1 (KIR3DL1) (clone DX9), and NK cells with the following KIR phenotypes were isolated: CD158a^/b^/e1^, CD158a^/b^/e1^, CD158a^/b^/e1^, and CD158a^/b^/e1^.

Tumor-specific activation of NK cells

Freshly isolated NK cells were suspended in complete medium at a concentration of 10^6/ml and incubated with an equal number of irradiated (30 grays) tumor cells for up to 20 h at 37°C and 5% CO_2. Stimulator tumor cells were restricted to the well-characterized leukemia cell lines U937, HL-60, and CTV-1, which were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) repository. Target cells in cytotoxicity assays included the NK-resistant RAJ1 and Daudi cell lines (obtained from the DSMZ cell bank), the breast cancer cell line MCF-7 (obtained from American Type Culture Collection), and primary leukemia, ovarian tumor, and breast tumor cells from patients attending the Royal Free Hospital, all of whom had given informed consent according to local

Research Ethics Committee approval. All cells were subjected to HLA typing as described above.

Analysis the immunophenotype of tumor-activated NK cells

Purified NK cells were mixed with an equivalent number of irradiated, PKH-26 labeled CTV-1 cells. Aliquots were removed at the time points indicated and labeled with anti-CD56 FITC and allophycocyanin-conjugated Abs to a variety of known NK associated Ags, washed, and analyzed by flow cytometry. CTV-1 cells were excluded from the analysis on the basis of forward angle light scatter and PKH-26 expression, and NK cells were positively included on the basis of forward angle light scatter and CD56 expression. The percentage of cells expressing each Ag was determined using cluster analysis and the relative fluorescence intensity was calculated as median channel log fluorescence of the univariate positive population

Production of lysates from CTV-1 cells

CTV-1 cell lysates were produced by two cycles of freeze-thaw treatment at $\sim$80°C and 37°C respectively. Lysates were incubated with 3 μg of Pulmozyme (Roche) per 5 × 10^6 cells at 37°C for 30 min to remove genomic DNA, followed by brief sonication to disrupt aggregates. Lysates from 10^6 CTV-1 cells were incubated with 0.5 × 10^6 freshly isolated NK cells as described above.

Cytotoxicity assay

Target cells were recovered from culture or cryopreservation and washed in HBSS before resuspension in 1.0 ml of PHK-26 labeling diluent at a concentration of 4 × 10^3/ml. A 4-μl aliquot of PKH-26 was added to 1.0 ml of labeling diluent and then added to the cell suspension for 2 min at room temperature. The labeling reaction was stopped by the addition of 1.0 ml of neat FCS for 1 min. Finally, the labeled cells were washed twice in complete medium and resuspended in complete medium at 10^6/ml. Fifty thousand PKH-26 labeled target cells in 100 μl of RPMI 1640 (10% FCS) were added to 400 μl aliquot of PKH-26 labeling diluent at a concentration of 4 × 10^3/ml. After 30 min of incubation, the labeled cells were washed twice in complete medium and resuspended in complete medium. Fifty thousand PKH-26 labeled target cells in 100 μl of RPMI 1640 (10% FCS) were added to 400 μl of effector cells (E:T ratio 1:1 or 5:1 as indicated in figure legends, because these are ratios that can be obtained in a clinical therapeutic setting without recourse to ex vivo proliferation) and pelleted at 200 × g for 1 min.

Cytotoxicity was measured in triplicate samples using a 4-h cytotoxicity assay at 37°C. After the incubation period the cells were resuspended in a solution of To-Pro-3 iodide (Invitrogen Life Technologies) in PBS (1 μM) and analyzed by flow cytometry. At least 10,000 target cells were acquired with 1024 channel resolution after electronic gating on red fluorescence, and the mean proportion of To-Pro iodide positive cells from the triplicate samples was determined. Background target cell death was determined from cells incubated in the absence of effector cells. Cell-mediated cytotoxicity was reported as the percentage of killing over background cell death averaged from the three samples (specific lysis): mean (percentage of cell lysis in test − percentage of spontaneous lysis).

### Table I. Patient characteristics

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Less than 5% spontaneous lysis of target cells was observed in these experiments. In some experiments the labeling strategy was reversed, with the effector cells being labeled with PKH-26 and the analysis of cell lysis being restricted to the PKH-26<sup>+</sup> fraction. This reversal confirmed that our initial findings were not due to an artifact of cell labeling.

**Analysis of the role of HLA:KIR interactions in tumor-activated NK (T-ANK) cell generation and lytic activity**

Purified NK cells from normal donors were selected on the basis of their HLA-A, -B, and -C type as KIR ligand matched or mismatched with CTV-1 cells. After overnight stimulation, the T-ANK cells were recovered and the lysis of KIR ligand-matched RAJI cells was determined as described above.

In two cases HLA-matched T-ANK cells from sibling allogeneic donors and presentation leukemic blasts (AML and chronic myeloid leukemia (CML)) from the patients were available. T-ANK and NK cells were isolated from the donor PBMCs and tested for in vitro cytotoxicity at E:T ratios from 1:1 to 10:1. Patients in clinical remission of AML after chemotherapy were tested in triplicate for autologous NK- and T-ANK-mediated lysis of their presentation leukemic blasts at E:T ratio of 1:1.

**Assessment of T-ANK lysis of normal HLA:KIR-mismatched hematopoietic cells**

T-ANK cells were generated from 10 normal donors and coincubated with PKH-26-labeled normal PBMCs from the same donor (autologous) and from KIR-ligand mismatched normal donors (allogenetic) at a 5:1 E:T ratio, and the specific lysis was measured. Additionally, T-ANK generated from PBMCs from 10 normal donors were incubated with HLA/KIR-mismatched bone marrow mononuclear cells at E:T ratios of 2:1, 10:1, and 20:1 for 4 hr before plating in semisolid colony-forming assays.

**Minimum requirements for T-ANK generation from restimulating NK cells**

To determine the minimum requirements for T-ANK generation, NK cells from 10 normal donors were coincubated with irradiated CTV-1 cells in three conditions. In the first, NK cells were physically separated from the CTV-1 in vitro (condition 1); in the second, NK cells were mixed with paraformaldehyde-fixed CTV-1, and in the third condition NK cells were mixed with irradiated CTV-1 in the presence of brefeldin A (5 μg/ml), which was washed out before the subsequent killing assay against RAJI target cells. The degree of RAJI cell lysis was compared with matched donor T-ANK. The results are presented as percentage change in the degree of lysis relative to the matched T-ANK cells to show the effect of each treatment.

**Production and purification of recombinant dimeric human CD69**

The extracellular domain of CD69 (residues 65–199) was amplified from cDNA (8) by PCR using primers introducing XhoI and HindIII restriction sites and a stop codon (CD69 forward, 5′-GCG CCT CGA CGA ATA CAA TTG CCC TCC AGG CCA ATA AT-3′; and CD69 reverse, 5′-GCC GAA GCT TAT TAT TTG TAA GAT TGT TTA CAA-3′). The PCR product was subcloned into the XhoI and HindIII restriction sites of the pET-19b(I) plasmid using a NdeI restriction endonuclease and the pET-19b(I)-pET-69. The DNA sequence that encodes the amino acid acceptor sequence for the Escherichia coli BirA protein ligase was added between the NdeI and XhoI sites of pET-19b(I)-69 with the following primers: 5′-CAT ATG CAT GGC GCG CTC GTG AAT GAA ATT GTG CAT GGC ACG CTG TTG GAT GGC ATG ATG CAT ATG-3′; and 5′-CTC GAG GGT TTC ATC ACA AGT GTC TAA AGG TCT GTC AAT-3′. DNA sequence was confirmed by automated sequencing using an ABI Prism 377 DNA sequencing kit.

Recombinant His-tagged human CD69 was expressed in BL21 (DE3) pLysS (Novagen) at 37°C. Cultures were grown in 1-liter batches in 2xTY medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. CD69 expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside after the culture had reached an OD<sub>600</sub> of 8.0. Cells were disrupted by multiple passages through a 16-gauge needle before centrifugation at 12,000 × g for 15 min at 4°C. The pellet was washed in isolation buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole). The protein was refolded by gradual removal of the urea through a linear gradient expanding from 10% refolding buffer to 100% wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole). This was achieved with 250 ml of buffer at 5 milliliter per minute using a HPLC system (Varian Technologies). After refolding, the protein was eluted with elution buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole).

Fractions were buffer exchanged into 10 mM Tris-HCl (pH 8.0) using PD10 columns (GE Life Sciences) and incubated with 2.5 μg of the BirA (avidity) per 10 nmol of the substrate at 30°C overnight following the manufacturer’s instructions. Excess biotin was removed and the protein concentrated by washing with 50 ml of HBSS in 10,000-Da molecular mass cutoff centrifuge tubes (Vivascreen) and assessed for rCD69 content by ELISA.

To assay CD69L expression by flow cytometry, cells were labeled with the biotinylated rCD69 and washed and incubated with streptavidin-PE (BD Pharmingen) before flow cytometric analysis. Additionally, to enhance the signal-to-noise ratio when screening normal cells, 5 μg of biotinylated rCD69 was conjugated to avidin-coated, yellow fluorescent 50-nm microbeads (Spherotech) by rotating incubation at 4°C for 40 min as described by Brown et al. and used to identify the ligand for 2B4 on NK cells. Protein bead conjugates were briefly sonicated to prevent aggregation and incubated with 10<sup>5</sup> target cells on ice for 60 min. Bound cells were washed with HBSS to remove any free avidin. The lysis was performed at a maximum of 40 events per second to prevent acquisition of coincident events. Binding of 5 μg of heat-denatured rCD69 was used as a negative control for each experiment.

**Blocking assay**

PKH-labeled target cells were preincubated with rCD69 or control reagent (6 μg per 10<sup>7</sup> cells) at 4°C for 30 min before set-up of the T-ANK cytotoxicity assay described above.

**Statistical analyses**

Data for comparative statistical analysis were assessed for normality (Gaussian distribution) and hence for comparable variance by F test (using GraphPad Prism version 4.0). Distributions with equal variance were tested for significant difference of their means by Student’s <i>t</i> test. Those with a significantly different variance were tested by Snedecor’s modified Student <i>t</i> test, which compensates for unequal variance. No data sets were non-Gaussian.

**Results**

**CTV-1 cells induce rapid up-regulation of CD69 on donor NK cells and prime lysis of NK-resistant targets**

NK cells isolated from normal healthy donors and coincubated with irradiated CTV-1 tumor cells synthesized CD69 within 60 min with maximal expression achieved within 6 h (Fig. 1A). The T-ANK cells lysed NK-resistant RAJI and Daudi cells in a 4-h assay. Individual donors showed heterogeneity of NK lysis of RAJI cells but, in all cases, the T-ANK cell lysis was significantly greater (<i>p</i> = 0.019). In contrast, preincubation with U937 or HL-60 lines showed no increase in RAJI cell killing (Fig. 1B). In addition, preincubation with RAJI, Daudi, K562 cell lines, and 20:20 primary AML cell samples had no effect on the lysis of NK-resistant lines RAJI or Daudi (data not shown). NK stimulation with CTV-1 was associated with low-level IFN-γ synthesis, but this was no greater than that induced by coincubation with HL-60, which did not induce T-ANK cell activity (data not shown).

**Missing self is not required for the generation of T-ANK cell activity**

CTV-1 cells are HLA-C type 2 homozygous and express HLA-Bw4 alleles. They can thus ligate KIR2DL1 (CD158a) and KIR3DL1 (CD158e1) on NK cells. Initially NK:CTV-1 cocultures before purification and refolding, pellets were resuspended in solubilization buffer (6 M guanidine hydrochloride, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole), passed through a 0.45-μm filter, and then loaded onto a 5-ml nickel-loaded Hitrap chelating column (GE Life Sciences) and pre-equilibrated with refolding buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 6 M urea, and 10 mM imidazole). The protein was refolded by gradual removal of the urea through a linear gradient expanding from 100% refolding buffer to 100% wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole). The fractions were exchanged into 10 mM Tris-HCl (pH 8.0) using PD10 columns (GE Life Sciences) and incubated with 2.5 μg of the BirA (avidity) per 10 nmol of the substrate at 30°C overnight following the manufacturer’s instructions. Excess biotin was removed and the protein concentrated by washing with 50 ml of HBSS in 10,000-Da molecular mass cutoff centrifuge tubes (Vivascreen) and assessed for rCD69 content by ELISA.

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were established with freshly isolated NK cells from donors who were HLA-matched or mismatched with CTV-1 with respect to the ligating KIR. It was thus possible to evaluate the contribution of “missing self” to the NK activation step. T-ANK cells were generated by CTV-1 from both HLA-C/KIR-matched and mismatched donors and there was no significant difference in the degree of specific lysis of KIR-ligand matched RAJI cells, although the T-ANK cells from matched donors showed greater heterogeneity (Fig. 2A). The degree of lysis was equivalent to that of matched lymphokine-activated killer cells (LAKs) after nonspecific activation with IL-2 (Fig. 2B).

The KIR phenotype of peripheral blood NK cells is not completely restricted by the HLA of the individual, and it is common for NK cells to lack appropriate KIRs for self MHC and even to express KIRs specific for HLA alleles absent from the individual (10). NK cells from normal donors were coincubated overnight with CTV-1 and phenotyped for the expression of KIR and CD69. It was readily apparent that CTV-1-induced NK activation was not restricted to KIR-mismatched NK cells, because cells expressing CD158a and CD158e1 showed equivalent levels of activation as NK cells from the same donors which lacked CD158a or CD158e1 but expressed CD158b, the ligand for which is absent from CTV-1 (Fig. 2C). To more precisely investigate the role of KIR in CTV-1-mediated activation and in the lysis of RAJI, NK cells were phenotyped and selected based on their KIR compatibility to the HLA of the CTV-1 stimulator cells as determined by flow cytometric sorting. Flow-sorted NK cell subsets were either incubated directly with CTV-1 cells or incubated overnight so that the anti-KIR Ab was shed from the NK cell and could not block KIR:HLA interaction. In both cases the NK cells expressing CD158a and CD158e1 showed equivalent lysis of RAJI cells as compared with CD158a/e1− NK from the same donors (Fig. 2D).

T-ANK cells lyse NK-resistant primary leukemias in vitro
T-ANK cells from allogeneic donors were capable of the lysis of primary AML cells of all French-American-British (FAB) types (Fig. 3A). These cells also lysed primary CLL cells at an E:T cell ratio of 1:1, although the level of killing was low. It was notable that the relatively NK-resistant breast cancer cell line MCF-7 was extremely susceptible to T-ANK cells, as were primary tumor cells

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**FIGURE 1.** Coincubation of normal donor NK cells with equal numbers of irradiated CTV-1 cells induces rapid and sustained expression of CD69 on the NK cells. A. Results are presented from 10 normal donors and expressed as the proportion of CD69+ cells within the CD56+ NK fraction (mean ± SD). B. NK cells from 25 normal donors stimulated with equivalent numbers of irradiated CTV-1, U937, or HL60 cells overnight were recovered from the coculture by flow cytometric or immunomagnetic cell sorting and coincubated at an E:T cell ratio of 1:1 with PKH-26 labeled RAJI cells for 4 h at 37°C and 5% CO2. Analysis of cytotoxicity was by flow cytometry as described (22, 23).

**FIGURE 2.** A. Comparison of T-ANK cell activity when donors’ NK were HLA-matched (m T-ANK) or HLA-mismatched (m/m T-ANK) (dotted line indicates mean). The difference was not significant according to Snedecor’s modified Student t test. B. Comparison of T-ANK cell lysis of RAJI cells with resting NK and IL-2-stimulated NK cells (LAK) from the same donors (solid line represents mean). C. Coexpression of KIRs and CD69 on CD56+CD3− NK cells after overnight incubation in medium alone (open bars), with irradiated CTV-1 cells (shaded bars), or with CTV-1 cell lysate (filled bars) (error bars show mean ± SD). D. Resting NK cells were flow sorted from three normal donors into CD158a+/CD158e1− and CD158a−/CD158e1+. These were stimulated overnight with CTV-1 cell lysates and their cytolytic activity was tested against RAJI cells in a 4-h assay (error bars show mean ± SD).
isolated from resected tissue from patients with breast cancer and ascites from patients with ovarian cancer (Fig. 3B). The lack of a requirement for HLA mismatch was confirmed in a study of two HLA-identical donors and their respective siblings with leukemia in the autologous setting. Two patients were selected from whom we had cryopreserved leukemic cells from their disease presentation and which were known to express HLA-A, -B, and –C (11). T-ANK cells from the HLA-identical sibling donor for patient 0100 effectively lysed cryopreserved CML blasts obtained from the patient at disease presentation. This lysis was apparent at an E:T ratio of 1:1 and was increased at increasing E:T ratios (Fig. 3C). In contrast, NK cells from the same donor were unable to lyse the CML blasts even at the highest E:T ratio of 10:1. The same was observed using T-ANK cells from an HLA-identical sibling donor for patient 0359, who presented with AML M2 and from whom presentation blasts had been cryopreserved.

Furthermore, T-ANK cells were generated from five patients in clinical remission from AML from whom presentation leukemic samples had been cryopreserved. Cases were selected on the basis that the presentation leukemic sample contained >95% AML blasts as determined by flow cytometry. All had been shown previously to express normal levels of HLA class I (>95% positive as determined by flow cytometry and with a median channel fluorescence intensity within 1 SD of the mean of normal peripheral blood myeloid cells). All five patients had functional NK cells as shown previously by lysis of K562 (7). The group included one AML M0, one AML M1, one AML M3, one AML M4, and one AML M7. Two patients had known low-level NK activity against their presentation disease, whereas three had no detectable autologous anti-leukemia activity (7). Cryopreserved aliquots of PBMCs were incubated overnight with irradiated CTV-1 at a ratio of two per NK cell and tested for lysis of freshly thawed autologous AML blasts (>95% pure) as described. The degree of T-ANK cell lysis was compared with freshly thawed NK cells from matched aliquots. All experiments were performed in triplicate. T-ANK cells from all five donors consistently induced significantly greater lysis of autologous AML blasts than the matched NK cells (p < 0.05) (Fig. 3D).

**T-ANK cell activity is restricted to tumor targets**

To investigate the tumor restriction of allogeneic T-ANK cells, we isolated NK cells from normal donors and either activated them with CTV-1 cells overnight or maintained them in medium. These T-ANK cells were then compared with NK cells from the same donor with respect to the lysis of normal autologous and allogeneic PBMC. Neither NK nor T-ANK cells lysed autologous PBMCs, nor did they lyse PBMCs from HLA-C mismatched normal donors (Fig. 4A). To determine the likelihood of bone marrow suppression by T-ANK cells, we established hemopoietic colony-forming assays with bone marrow from five normal donors and added T-ANK cells from HLA-C mismatched normal donors at increasing ratios. CFU-granulocyte-macrophage (CFU-GM), burst-forming unit-erythrocyte (BFU-E), and CFU-granulocyte, erythrocyte, monocyte, and megakaryocyte (MEGG) were not affected by coincubation with HLA-mismatched T-ANK cells even at E:T ratios of 20:1 (Fig. 4B). In contrast, T cells derived from the same donors suppressed growth in all cultures by >70% (data not shown).
Cell:cell contact and protein secretion are required for the NK priming

CTV-1 cells did not secrete IL-2, IL12, IL-15 nor IFN-γ as determined by an ELISA of culture supernatants. To investigate whether some other cytokine was responsible, Transwell cultures were established with NK cells in the upper chamber and CTV-1 cells below. All T-ANK cell activity was suppressed (Fig. 5A). In contrast, fixation of the CTV-1 cells with paraformaldehyde did not prevent NK priming. The addition of brefeldin-A to the coculture significantly reduced the subsequent T-ANK cell lytic activity (\( p < 0.03 \)), suggesting that T-ANK cell lytic function required ligation of a newly expressed receptor on the surface of the T-ANK cells that was absent from the resting NK cells. The addition of brefeldin-A to the coculture significantly reduced the subsequent T-ANK cell lytic activity (\( p < 0.03 \)), suggesting that T-ANK cell lytic function required ligation of a newly expressed receptor on the surface of the T-ANK cells that was absent from the resting NK cells. Cell surface phenotyping of T-ANK cells showed no increase in the frequency or intensity of expression of a wide range of known activating receptors or a significant reduction in the expression of inhibitory receptors that might lower the threshold for NK lysis (Fig. 5, B and C). However, CD69 expression was induced consistently while CD16 expression was reduced markedly (Fig. 5B). In contrast, NK priming with CTV-1 did not increase the levels of intracellular perforin or granzyme B, nor did it affect the surface expression of CD56 or CD107a, the increased expression of which is associated with increases in intracellular perforin (Fig. 5C). CD69 up-regulation was blocked in the presence of brefeldin A (data not shown).

The activating ligand on CTV-1 is unknown

CTV-1, HL60, and U937 share the expression of many surface molecules, but CTV-1 uniquely expresses CD7, CD11a, and CD38 strongly. The blocking of CD11a marginally reduced the degree of CD69 up-regulation on NK cells after coincubation with CTV-1 as did a blockade with anti-CD7 or anti-CD38 to a lesser extent, although none of the mAbs either alone or in combination inhibited T-ANK cell generation (data not shown). To further investigate whether CD11a and/or CD54 are involved in the CTV-1-mediated NK activation, P815 cells were coated with anti-CD11a, anti-CD54, or a combination of the two and cocultured at varying stimulator-to-responder ratios with sorted NK cells. In contrast to irradiated CTV-1 cells, the irradiated P815 showed no ability to induce T-ANK cell activity irrespective of the coating
We have shown previously that CD69 expressed on the activated NK cell caps at the immunological synapse with an autologous AML cell (7), and we confirmed this at the synapse between T-ANK and RAJI cells (Fig. 6A). This finding implies that a CD69L is expressed on T-ANK-susceptible tumor cells. The identity of this CD69L is currently unknown.

We produced a soluble recombinant human CD69 protein that was biotinylated and conjugated to streptavidin-PE and to green fluorescent nanoparticles as previously described (9) to generate multimeric reagents. The expression of CD69L was demonstrated using a nanobead reagent on RAJI cells by confocal microscopy (Fig. 6B). This expression was confirmed with the streptavidin-PE reagent analyzed by flow cytometry (Fig. 6C). CD69L expression was absent from NK-sensitive K562 cells (Fig. 6C), normal T cells (Fig. 6D), and normal NK cells (Fig. 6D), all from healthy donors (n = 5). None of eight EBV-LCL from normal donors bound rCD69 (Fig. 6C). CD69L expression was detected on other cell lines susceptible to T-ANK cell-mediated lysis, including Daudi cells and MCF-7 cells (Fig. 6C), whereas ARH77 cells, which were poor T-ANK cell targets, showed no binding of the rCD69 reagent (Table II). CD69L was not detected on normal PBLs. The rCD69/nanobead reagent showed higher signal-to-noise ratio than the streptavidin-PE reagent and should, therefore, lead to more sensitive detection of CD69L. By using this multimeric reagent we confirmed the lack of expression of CD69L on normal lymphocytes (Fig. 6D). Three of 10 primary AML samples that were susceptible to allogeneic T-ANK cell-mediated lysis also bound rCD69. Three AML samples that were resistant to T-ANK cell killing also bound rCD69, implying that additional molecules are required for complete triggering of the lytic machinery.

**CD69 is critical for tumor-restricted killing by T-ANK cells**

To establish the role of the CD69:CD69L interaction in T-ANK cell activity, we sorted the CD69+ T-ANK cells after CTV-1 stimulation from the CD69− cells before a RAJI lysis assay. The CD69+ fraction mediated 83.7% of the activity of unfractionated T-ANK cells, whereas the CD69− NK cells showed 5.5% (Fig. 7A). The critical role of CD69 in T-ANK cell triggering was confirmed by the inhibition of RAJI cell lysis in the presence of rCD69. Preincubation of RAJI cells with rCD69 significantly reduced the degree of RAJI cell lysis almost to the level of lysis by resting NK cells. This effect was not observed when RAJI cells were preincubated with BSA or heat-denatured rCD69 (Fig. 7B). As expected, rCD69 did not block lysis of K562 either by resting NK cells or by T-ANK cells (Fig. 7C).

The addition of rCD69 to assays of autologous T-ANK cell-mediated lysis of AML as described above significantly decreased lysis in five of five cases (p < 0.04) (Fig. 3D), confirming that the role of CD69:CD69L interactions is not restricted to allogeneic responses to cell lines and is likely to be clinically relevant. All five AML samples expressed CD69L.

### Table II. Tissue distribution of CD69L expression

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>CD69L Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal T cells</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Normal B cells</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Normal NK cells</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>RAJI</td>
<td>Burkitt’s lymphoma</td>
<td>Positive</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt’s lymphoma</td>
<td>Positive</td>
</tr>
<tr>
<td>K562</td>
<td>Erythroleukemia</td>
<td>Negative</td>
</tr>
<tr>
<td>ARH77</td>
<td>Myeloma</td>
<td>Negative</td>
</tr>
<tr>
<td>Jurkat</td>
<td>T cell lymphoma</td>
<td>Negative</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Metastatic breast tumor</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**CD69 on T-ANK cells binds to CD69 ligand on tumor targets**

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Discussion

In this study, we have shown that human NK cells can be specifically primed by a tumor cell to lyse other tumor cells even in the presence of inhibitory ligands. Furthermore, this lysis is restricted to tumor cells because normal hemopoietic cells, even those lacking the relevant inhibitory ligands, are not affected, and it operates at extremely low E:T ratios. It has been shown previously that the lack of KIR-mediated inhibition is not sufficient per se to initiate NK lysis of primary leukemias; Ruggieri et al. (12), for example, found no protection from relapse in recipients of KIR-mismatched stem cell transplants when the leukemic cells lacked expression of LFA-1. The issue of whether lack of inhibition is a requirement for NK-mediated lysis is more controversial. The transfection of relevant HLA-C molecules into NK-sensitive P815 or K562 cells has been shown to be sufficient to render those cells resistant to lysis (3, 13). In contrast, we and others have demonstrated autologous NK cell-mediated lysis of primary acute myeloid leukemia cells that expressed normal levels of HLA-class I molecules (4, 7), and others have shown the retention of KIR-ligating HLA molecules on leukemic blasts at normal or mildly elevated levels (11, 14). One may hypothesize that P815 cells, being of murine origin, lack sufficient “activating” ligands for human NK cells to overcome inhibition signals from transfected HLA-C molecules. K562 cells, though of human origin, may also lack activating ligands because they failed to bind the rCD69 reagent in our experiments and similarly failed to bind recombinant NCR proteins (15); nor does CD69 act as a trigger for the lysis of these cells. This finding is informative in the light of the data from Bryceson et al. (5), because they have shown that resting NK cells cannot be triggered to induce lysis through the ligation of multiple NCR, and yet these same cells will spontaneously lyse K562 cells. This implies that the lysis of K562 cells by resting NK cells either requires only triggering or that K562 cells provide both activating and triggering signals. The fact that preincubation of NK cells with K562 does not induce T-ANK cell activity would argue for the former hypothesis, and the lack of inhibition of NK-mediated lysis by rCD69 supports the contention that lysis of K562 does not require activation, because CD69:CD69L interactions are not involved.

Uniquely, our separation of the activation step from the lytic event in this model system has shown that the KIR-mediated inhibitory signals can be overcome by interactions with tumor cells and that these activated NK cells remain insensitive to inhibitory KIR signals from resistant cells. Our findings support those of Bryceson et al. (5), who showed that, in contrast to NK clones or IL-2 activated NK cells, resting NK cells require ligation of at least two receptors to trigger lysis, although the separation of these into activating vs triggering ligands was not discussed. They showed that up-regulation of CD107a was achieved by concomitant ligation of...
NKG2D and 2B4 and that this was associated with triggering of lytic machinery (5). In our experiments, stimulation of resting NK with the CTV-1 cells did not induce CD107a expression, arguing that this stimulation represents a separate activation step. Bryceson et al. (5) showed that IL-2-activated NK cells required only one trigger ligand to initiate cytotoxicity, and we believe this is analogous to the state of our T-ANK cells.

It was intriguing that, of all of the tumor cell lines and primary tumors studied, only CTV-1 was able to induce T-ANK cell activity. We currently hypothesize that NK-sensitive tumor cells provide both priming and triggering signals. In contrast, NK-resistant tumors evade NK-mediated lysis through the lack of priming ligands or the lack of trigger ligands for primed NK cells. The prototypical NK target cell line K562 is also sensitive to T-ANK cell lysis, but the level of lysis is no greater than that induced by resting NK cells (Fig. 7C), implying that K562 both activates and triggers NK cells. In contrast, cells such as RAJI or Daudi fail to prime NK cells but are susceptible to lysis by NK cells primed by IL-2 or by an appropriate tumor cell such as CTV-1. CTV-1 evades NK-mediated lysis by failure to trigger primed NK cells. This was supported by the fact that CTV-1 cells are resistant to LAK- and T-ANK cell-mediated killing. Thus, resting NK cells cocultured with CTV-1 are primed but not triggered such that they are capable of lysing NK-resistant cells that express one or more triggering ligands. From our investigations it appears that the failure to trigger lysis by primed NK cells is a rare property of tumor cells.

These data add another layer of complexity to the currently accepted understanding of human NK cell activation. The data may explain some of the disparity in the literature regarding the role of NK cells in graft-vs-leukemia activity after an allogeneic hematopoietic stem cell transplant where the NK cells that appear in the peripheral blood in the first few months after transplant lack KIR. These cells do, however, express CD94/NKG2A, CD94/NKG2D, and the natural cytotoxicity receptors of the Ig superfamily.

The resistance of normal hematopoietic cells to lysis by T-ANK cells, even in the absence of relevant KIR-ligating HLA, implies that the tumor cells (T-ANK cell targets) express a tumor-restricted ligand that is responsible for T-ANK cell triggering. The lack of T-ANK cell generation in the presence of brefeldin A confirmed that the signaling molecule for T-ANK cell-mediated lysis was newly expressed upon coincidence with the stimulatory tumor cells. Of the known NK cell triggering molecules, only CD69 was up-regulated during the preincubation and, because we have shown previously that CD69 on NK cells caps at the synapse with AML blasts (7), we investigated its role in T-ANK cell activity.

Many hematopoietic cells express CD69 upon activation. Ligation of CD69 on human NK cells, in contrast to T cells, has been shown to initiate tumor cell lysis (16). Conversely, murine data imply that CD69 ligation is inhibitory to NK-mediated lysis because CD69 knockout mice show enhanced antitumor activity (17), and mAb blockade of CD69 on murine NK cells increases their lytic activity (18). There is good reason to propose that CD69 is a tumor-specific triggering receptor on human NK cells because of its similarity to NKG2D, another homodimeric C-type lectin that binds to MICA-, MICB-, and UL16-binding proteins and can be used to target malignant cells while sparing normal hematopoietic cells (19). By producing a recombinant dimeric human CD69 protein, we confirmed that CD69 on activated NK cells is the predominant trigger molecule for T-ANK cell cytotoxicity of NK-resistant cell lines and one of the triggers of lysis of primary AML cells. Lysis of K562 by NK or T-ANK cells does not involve CD69:CD69 interaction. This is supported by the observation that T-ANK:RAJI cell conjugation led to Syk activation within the T-ANK cells (data not shown), a phenomenon known to be associated with CD69-mediated signaling (20) and which implies that the CD69:CD69L interaction is not simply increasing cell-cell adhesion. It is noteworthy that the generation of T-ANK cell activity and up-regulation of CD69 coincided with the loss of expression of CD16, which is analogous to the phenotype of leukemia-reactive NK cells recently described elsewhere (21). We have no evidence that T-ANK cells differ phenotypically from conventional LAK cells but believe that these are the first data to show that resting NK cells can receive a priming signal from a tumor cell rather than a cytokine.

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Disclosures
The authors have no financial interest of interest.

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killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood 105: 3615–3622.


