Leukemic Priming of Resting NK Cells Is Killer Ig-like Receptor Independent but Requires CD15-Mediated CD2 Ligation and Natural Cytotoxicity Receptors

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J Immunol published online 14 November 2011
http://www.jimmunol.org/content/early/2011/11/14/jimmunol.1101640
Leukemic Priming of Resting NK Cells Is Killer Ig-like Receptor Independent but Requires CD15-Mediated CD2 Ligation and Natural Cytotoxicity Receptors

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Resting human NK cells require at least two activating signals before commitment to cytokine secretion and/or target cell lysis (1). We demonstrated that these two signals can be divided into discrete “priming” and “triggering” events, with the priming signal being provided either by activating cytokine, such as IL-2, or by conjugation to a tumor cell expressing an appropriate intensity and combination of ligands for priming receptors (2).

CD2 ligation is known to be one of the mechanisms by which resting NK (rNK) cells are primed to activate (1). It was reported previously that a novel ligand for CD2 is structurally associated with CD15, such that some anti-CD15 mAbs sterically block the CD2L site and are able to block NK-mediated lysis of otherwise susceptible target cells, including the prototypical NK target, K562 (3). It can be easily envisaged that tumor cells may evolve at least two strategies for evasion of NK-mediated lysis: lack of priming ligands or lack of triggering ligands. Because most known NK-resistant tumor cell lines are susceptible to IL-2–primed NK (pNK) cells (lymphokine-activated killer cells), one must presume that these cell lines express NK-triggering ligands but lack the priming ligands. We previously reported a single cell line that primes rNK cells but fails to trigger the secretion of cytokines or the lytic activity (2). This NK cell priming requires cell–cell contact and conjugate formation and may be used to dissect the tumor-induced changes required for susceptibility to NK cell lysis. The original leukemic cell line identified by our group was the CD15+ CTV-1 cell line, an acute lymphoblastic leukemia (ALL) with myeloid features.

CTV-1 cells express numerous known ligands to molecules expressed on rNK cells. These include CD58 and CD48, which are the common ligands for CD2 and 2B4 on NK cells. CTV-1 also expresses high levels of CD15. Because CD58 blockade had no effect on NK cell activation, we hypothesized that the CD15 on the CTV-1 included the CD2L site and that this was responsible for the priming of rNK cells. Furthermore, we hypothesized that induced expression of CD15 on an NK-resistant tumor cell would render it susceptible to lysis by rNK cells. Although the natural ligands for natural cytotoxicity receptors (NCRs) have not been fully identified, their innate high surface expression on rNK cells suggests that they may also have a key role in the priming process.

Materials and Methods

Cell lines

All cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) or from Laboratory of the Government Chemist (London, U.K.) and cultured as recommended by the repository. The CTV-1 line was originally reported to be of myeloid origin but was recently shown to be an ALL with myeloid features and that expresses CD15. The SEM line is another CD15+ ALL line with the t(4;11) translocation. MOLT-16 and PF-382 are both CD15−.
ALL lines, MV-411 is a biphenotypic CD15+ (4:1:1) acute myelomonocytic leukemia. The RAJI line is a B cell line derived from a non-Hodgkin’s lymphoma and is one of the prototypical NK-resistant lines. K562 cells are the prototypical NK-sensitive line derived from an erythroblastoïd leukemia and that lack expression of HLA molecules. All were maintained in continuous suspension culture and harvested in exponential-growth phase prior to use as stimulator or target cells. DU145 cells were derived from prostate tumor epithelial cells, cultured as adherent cells, and harvested by trypsinization at confluence.

**Cell culture reagents**

All cell cultures were maintained in complete medium (CM) consisting of RPMI 1640 supplemented with 10% v/v FCS, penicillin (100 IU), and streptomycin (100 IU) (all supplied by Invitrogen, Paisley, U.K.).

**Immunophenotyping**

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

**Isolation of human NK cells**

CD56+ cells were purified from PBMCs or PBMC/stimulator cell cocultures by direct immunomagnetic separation with a CD56 Multisort kit. Target cells were recovered from suspension culture and washed in HBSS and resuspended in CM. Immunoselection with anti-CD56 microbeads and resuspended in CM at a concentration of 10^6/ml. A 4-µl aliquot of PKH-26 was added to 1.0 ml 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 neat FCS for 1 min. Finally, the labeled cells were washed twice in CM and resuspended in CM at 10^6/ml. Fifty thousand PKH-26-labeled target cells in 100 µl RPMI 1640 (10% FCS) were added to 400 µl effector cells (E:T ratio: 1:1 or 5:1) and pelleted at 200 X g for 1 min.

**Cytotoxicity assay**

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) 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 PKH-26 positivity, 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 percentage killing over background cell death averaged from the three samples (specific lysis): mean (% cell lysis in test – % spontaneous lysis).

**Statistical analyses**

Data for comparative statistical analysis were assessed for normality (Gaussian distribution) and then for comparable variance by the F-test (using GraphPad Prism v4.0). Distributions with equal variance were tested for significant differences in their means by the Student t test. Pairs of NK–pNK cells were tested for significant differences in their means by the paired t test. Distributions with significantly different variance were tested by the Snedecor modified t test, which compensates for unequal variance. No data set was non-Gaussian.

**Activation of NK cells in the presence of NCR blockade**

The CD69 surface molecule is upregulated on resting human NK cells by CTV-1 stimulation and can be used as a marker of successful priming. Matched pairs of rNK and pNK cells were immunophenotyped for CD69 (to assess NK activation), C-type lectin receptors (NKG2D, NKp80), and λ- and α-superfamily (SF)-like NCRs (NKp30, NKp44, and NKp46). The proportion of cells expressing the specific Ag and its relative density of expression (median channel fluorescence [MedCF]) were compared.

**Isolation of CD15-transduced RAJI cells**

The cDNA encoding human α1,3-fucosyltransferase IV (FUT4), which is required for the expression of CD15 (4), was blunt ended PCR cloned into pseudotyped (p)Lenti6/V5 (Invitrogen) from lymphocyte cDNA (a gift from Dr. Steve Hart) using the forward primer 5′-ATG GGC TCG CCG TGG GGC TCG CCG AC-3′ and 5′-AGT GGC GAG CTT GTG CCA CGG GTT-3′ and verified by sequencing to give pLenti6/CDS. p-Vesicular stomatitis virus-G was produced from 293T cells after transfection with pCMVDR8.91 (5), p-vesicular stomatitis virus-G (6), and pLenti6/CDS at a ratio of 3.5:1:75:5 using calcium phosphate. Twenty-four hours posttransfection, serum-supplemented medium was replaced with serum-free medium, and the virus was harvested and concentrated by calcium precipitation 48 h later. Viral supernatant was concentrated to a pellet and resuspended in 1 ml buffer (100 mM EDTA, 50 mM NaCl, 0.2% BSA [pH 6.5]). A total of 10^7 RAJI cells was cultured with 5 m × 10^7 PFU for 6 h before being pooled, washed, and resuspended at 5 × 10^5/ml in fresh RPMI 1640 + 10% FCS in a flask. CD56 expression was assessed by 48 h postinfection by flow cytometry.

**Results**

CD15+ leukemia cells prime lysis of NK-resistant tumor cells

Resting NK cells that had been cocultivated with NK-resistant CD15+ tumor cells (CTV-1, MV-411, SEM), but not CD15-ve
cells (MOLT-16, PF-382), lysed RAJI cells (Fig. 1A) and were capable of lysing a variety of NK-resistant tumor cells of different lineages, including the RPMI8226 myeloma line, the ARH77 plasmacytoma line, and the DU145 prostate line (Fig. 1B). Resting NK cells were >95% CD56dim, and the majority coexpressed CD16. In accordance with our previously published findings, coinoculation with CTV-1 led to significant shedding of CD16 (Fig. 1C). The NK-priming cell line CTV-1 expresses high levels of CD15. We coincubated rNK cells with CD15+ cells prelabeled with the stable cell membrane dye PKH-26. Within 24 h, the pNK cells expressed CD15 and detectable PKH-26 (Fig. 1D), indicating cell–cell transfer rather than induced expression. This is akin to the transfer of MICA, which was reported previously (7), suggesting that CD15 is an important constituent of the NK–CTV-1 immune synapse.

**NK priming is mediated by CD15-mediated ligation of CD2**

We hypothesized that the two signals needed to initiate lysis could be delivered sequentially, because the priming tumor cell was always absent from the NK–tumor cocultures. We screened CTV-1 cells for potential NK-activating ligands with 58 mAbs (data not shown) and identified five candidate molecules: CD58, CD48, CD38, CD15, and CD11a/CD18 complex. Of these, only CD15 significantly (p < 0.01) suppressed the activation of NK cells following CTV-1 coincubation (Fig. 2A), as measured by CD25 and CD69 expression.

The ability of anti-CD15 to block NK priming was tested in cytotoxicity assays. The prototypical NK-resistant RAJI cell is sensitive to NK-mediated lysis after priming through contact with CTV-1. Resting human NK cells were coincubated with CTV-1 in the presence or absence of blocking Abs for 20 h and tested in 4-h killing assays against RAJI cell targets. We used two anti-CD15 mAbs, clone LeuM1 (BDIS, Oxford, U.K.) and clone MEM158 (Serotec, Oxford, U.K.), the former having been reported to bind a site within CD15 that excludes the CD2L site (3). Only pre-incubation with anti-CD15 clone MEM158 significantly reduced the pNK lytic activity (Fig. 2B), indeed to a level below that of anti-CD49f, the positive inhibitory control that we previously showed to suppress IL-2–primed NK lysis of lymphoma cell lines by 30–50% (8). The role of CD2 as the NK-associated ligand for CD15 was confirmed by the blockade of NK priming by anti-CD2 (OKT11; American Type Culture Collection). Anti-CD2 Ab OKT11 significantly reduced the CTv-1–mediated priming of NK cells, as measured by CD25 expression (Fig. 2A) and by RAJI cell lysis (Fig. 2B).

**CD2-mediated priming of NK cells is unaffected by their degree of functional maturation**

CD57 is a marker of fully functionally differentiated NK cells (9), and it was hypothesized that the requirement for CD2-mediated priming might differ between CD57+ and CD57− NK subsets. CD2 is expressed on >80% of resting human NK cells, and its...
presence is unrelated to the degree of functional differentiation, as determined by CD57 expression. The ability of CTV-1 cells to prime rNK cells through CD15–CD2 interaction was equivalent on both CD57+ and CD57− NK cells, although it was notable that NK priming via CD2 led to a consistent increase in the proportion of CD57+ NK cells in culture (mean increase, 23.6%, SD 9.75, \( p = 0.024 \)) and a consistent, but modest, increase in CD57 expression (mean increase in MedCF, 6.14%, SD 4.28, \( p = \text{NS} \)).

Transfection of CD15 into NK-resistant cells provides the priming signal

Prima facie evidence of the role of CD15 was achieved through transfection of RAJI cells with the cDNA for FUT4. Transfection of FUT4 into RAJI cells led to expression of CD15 within 48 h (Fig. 3A) and rendered the cells susceptible to lysis by rNK cells (Fig. 3B). This was blocked effectively by addition of a saturating concentration of anti-CD15 mAb (clone MEM158) or anti-CD56 (clone NCAM16.2; BDIS). Normal RAJI cells proved resistant to lysis by rNK cells, whereas the CD15-transfected RAJI cells showed a high level of susceptibility to NK-mediated lysis. This was blocked by anti-CD15 mAb, but not by the control anti-CD56 mAb. K562 cells were predictably sensitive to lysis by rNK cells, but this too was blocked by addition of anti-CD15 mAb, confirming the role of CD15 in NK priming. All data are mean ± SD of three experiments.

CD2 ligation via the CD15-associated ligand induces synthesis of IFN-γ via the LAT–STAT signaling cascade

Conjugation of rNK with CTV-1 leads to rapid shedding of extracellular CD16 (Fig. 1C), and we hypothesized that this might facilitate the interaction of the cytoplasmic CD16/CD3ζ complex with the intracellular domain of CD2, because CD3ζ is required for CD2-mediated signaling in NK cells (10). As shown in Fig. 4A, coincubation of rNK cells with CTV-1 cells induced rapid phosphorylation of CD3ζ. CD2 ligation was shown to lead to phosphorylation of linker for activation of T cells (LAT) (11), and flow cytometric analysis showed phosphorylation of LAT in
the absence of ZAP70 phosphorylation (Fig. 4B), confirming ligation of CD2 by CD2L within CD15 on the CTV-1 cell. Phosphorylation of LAT is thought to be via phosphorylation of ZAP70, but we were consistently unable to demonstrate p-ZAP70 upon CTV-1 coculture or following cross-linkage with the anti-CD2 Abs available to us (data not shown). In contrast, cross-linkage of CD3 on T cells led to rapid and sustained p-ZAP70 (data not shown). This suggested that CD2-mediated LAT phosphorylation in NK cells may be independent of p-ZAP70.

Coincubation of CTV-1 cells with nNK cells led to rapid phosphorylation of Stat5 (Fig. 4C) in the NK cells and was associated with upregulation of CD25 (Fig. 5A) and CD69 (Fig. 5C) and increased synthesis of IFN-γ within 4 h (Fig. 5F), which was not achieved with IL-2 stimulation until 48 h of culture. IFN-γ and TNF-α were consistently induced by CTV-1 priming, whereas there was no detectable increase in the production of granzyme B or perforin at this priming stage (Fig. 5H).

**CD2-mediated and IL-2-mediated activation of nNK cells operate by different pathways and have different physiological outcomes**

In contrast to the CD3ζ–LAT–Stat5 pathway activated by CD2 ligation (12), IL-2 is known to activate NK cells via MAPK 1/ extracellular signal-related protein kinase (13), but upregulation of CD69 and IFN synthesis take a minimum of 48 h (Fig. 5D). Upregulation of CD25 expression following IL-2 stimulation was also significantly slower than that induced by CD2 ligation by CTV-1, and the proportion of activating NK cells was consistently smaller (Fig. 5A).

**Blocking of lectin-type NCRs restricts NK priming**

CD69 expression is a hallmark of NK priming. Within 16–20 h of coincubation with CTV-1 cells, the proportion of CD69+ NK cells increased significantly (17.17–61.75%; p = 0.0061), as did the intensity of CD69 expression (MedCF increased from 6.68 to 32.44; p = 0.013), compared with matched, nonstimulated NK cells. NKp80, NKp30, and NKp44 were unaffected by tumor priming, whereas significant downregulation of NKp2D and NKp46 was a consistent finding (Fig. 6).

Blockade of C-type lectin receptors NKp2D and NKp80 significantly suppressed the proportion of CD69+ NK cells (p = 0.012) and the intensity of CD69 expression (p = 0.0244) induced by CTV-1 priming. In contrast, blockade of Ig-SF–like receptors NKp30, NKp44, and NKp46 had no significant effect on the proportion or intensity of CD69 expression, nor did it enhance the effect of C-type lectin receptor blockade (Fig. 7).

**CD2-mediated NK cell priming is stable after removal of the priming signal**

Previously published research established that IL-2–activated NK cells rapidly return to the nonactivated state upon removal of IL-2. We stimulated lymphocytes from 10 normal donors with a lysate of CTV-1 cells for 20 h at a nominal CTV-1/NK cell ratio of 2:1. Fig. 8A shows the NK cells (R2), the NK T cells (R3), and the lysate (R4). NK cells were then directly isolated by immunomagnetic selection with anti-CD56 microbeads. After immunomagnetic selection, the resulting pNK cell preparations were shown to be free of CTV-1 contamination by flow cytometry (Fig. 8B), because the region containing the lysis events (R4) was empty. The CD56+ cell fractions were then split into two fractions: one was tested immediately for functional RAJI lysis, whereas the second was cryopreserved in vapor-phase nitrogen before thawing and retesting for RAJI lysis. The degree of RAJI cell lysis mediated by freshly isolated tumor-primed NK cells was not significantly different from matched cells that were cryopreserved in nitrogen vapor for 14 d (Fig. 8C), despite the complete absence of CTV-1 lysate stimulation for >2 wk.

**Discussion**

The regulation of human NK-mediated lysis of tumor cells involves a myriad of cell surface receptors on both the NK and tumor cells providing inhibitory and activatory signals. Lysis occurs when the combination of activation signals overcomes any inhibitory signals provided by ligands on the target cell. The principal ligands for inhibitory molecules appear to be the HLA class I Ags, and susceptibility of tumors to NK-mediated lysis was shown to be related to the degree of expression of these molecules (14). In contrast, the nonsusceptibility of the B lymphoma cell line RAJI to NK-mediated lysis has been attributed to its expression of all of the known classes of ligands for NK-inhibitory molecules. Nonetheless, RAJI cells are susceptible to lysis by IL-2 or IL-15–activated NK cells, implying that the trigger ligands for NK activity are present at sufficient levels if the inhibitory signals can be overcome.

Bryceson et al. (1) used the murine mastocytoma cell line P815 to test NK-activating ligands in reverse-cytotoxicity assays using resting, freshly isolated human NK cells, rather than NK
lines or clones. In line with the previous report from Warren et al. (3), these data confirmed that the lack of NK inhibition by HLA is not sufficient to trigger lysis; P815 cells do not express HLA but are resistant to human NK lysis. Furthermore, with the exception of CD16, the other known NK-triggering ligands require at least one coligation event to trigger cytokine secretion and/or lysis. The group did not investigate whether the two signals required needed to be delivered simultaneously or could be provided sequentially.

Previous work from our group showed that an NK-resistant cell line that is also resistant to lysis by IL-2–activated NK cells is able to prime rNK cells to lyse RAJI cells (2); in this study, we extended that finding to two other NK-resistant tumors.

NK cells are an extremely important part of human defense against tumorigenesis and appear to play a critical role in ongoing tumor surveillance in healthy individuals (15). The most fundamental control mechanisms for NK cells may not be inhibition through self-MHC but may be through a requirement for sequential or simultaneous activation signals akin to the costimulation and triggering of T cells. The data from Bryceson et al. (1) and our own observations (2) support this hypothesis; to our knowledge, the results presented in this article are the first to demonstrate the critical importance of the expression of a ligand for NK costimulation on a tumor cell in the initiation of lysis.

More than 10 years ago, Warren et al. (3) described a moiety within the CD15 epitope of Lewisx that is a ligand for CD2 and that is present on the prototypical NK target cell, K562. Furthermore, they found that its expression on K562 was required for NK-mediated lysis; the absence of HLA class I expression was not sufficient to trigger lysis. CD2 is a known costimulatory priming molecule on rNK cells (1).

Evidence to support the hypothesis that CD15 is involved in the ligation of CD2 was provided by the Ab-blocking experiments and the demonstration of phosphorylation of CD3ζ. In contrast to CD2 on T cells, CD2 on NK cells is not constitutively associated with CD3ζ. Cytoplasmic CD3ζ is bound to CD16 in NK cells (9), which, presumably explains the unique ability of CD16 to activate and trigger resting human NK cells (1).

One of the known outcomes of CD2 signaling is the phosphorylation of STAT5, which can lead to IFN-γ synthesis in NK cells (12). Indeed, p-STAT5 was detectable within 5 min of coincubation of rNK cells with CTV-1, which was followed by IFN-γ synthesis.

Although CD15-associated CD2L is required for NK-mediated lysis of K562 by rNK cells, it is not itself sufficient to prime NK cell activity, because resting, normal human monocytes that express CD15 are unable to stimulate NK activity. Thus, some form of tumor-restricted signal(s) must be delivered to rNK cells.

**FIGURE 5.** NK activation following CTV-1 or IL-2 stimulation. Resting NK cells were primed overnight with CTV-1 cells at a 1:2 ratio or for 48 h with IL-2 (100 IU/ml) and then labeled for expression of CD25. A, Tumor priming consistently induced greater expression of CD25 than did incubation with IL-2. B–G, Parallel cultures were labeled for surface expression of CD69 and intracellular IFN-γ. Resting NK cells (B, E) stimulated with CTV-1 upregulated CD69 within 4 h (C) and synthesized intracellular IFN-γ within 6 h (F). In contrast, IL-2 stimulation for 48 h induced less CD69 (D) and negligible IFN synthesis (G). The vertical cursor lines in each graph represent maximum fluorescence of the specific isotype-matched negative control. H, Primed NK cells (●) showed consistent synthesis of IFN-γ and TNF compared with rNK cells (○) while not inducing synthesis of either granzyme B or perforin. Horizontal lines in A and H represent mean values.
in the presence of costimulation to prime them to lyse tumor cells that present appropriate triggering ligands. Many candidate molecules for providing tumor-restricted signals have been proposed, including heat shock proteins, lectins, and complex carbohydrates, but the essential ligands remain elusive. CTV-1 cells would appear to be a valuable tool in the further dissection of these ligands, because it is clear that the simple lack of ligands for inhibitory receptors and the presence of appropriate adhesion molecules is not sufficient for NK cell cytotoxicity of tumor cells.

In this article, we demonstrated that C-type lectins are the predominant NCRs involved in the process of NK cell priming by tumor cells, whereas the Ig-SF–like receptors have little or no role in the priming process. This may partially explain the evolutionary conservation of these two distinct families of NKRs.

The maintenance of the primed state in NK cells following removal of the priming tumor cell lines is a unique observation that contrasts sharply with the requirement for continuous cytokine exposure for the generation and maintenance of lymphokine-activated NK cells. In this article, we demonstrated that NK cells can be primed by a short period of coincubation with relevant NK-resistant tumor cells, which can then be removed with no effect on the primed state. These pNK cells retain the ability to lyse NK-resistant tumors after cryopreservation and thawing, allowing easy translation to clinical application, because the cells can be prepared remotely and quality assured before release. We just completed a clinical trial of tumor-primed NK cells in acute myelogenous leukemia patients on this basis.
Acknowledgments
We thank Bob Balderas at BD Biosciences for the generous supply of reagents for the phosphoflow studies.

Disclosures
M.W.L. is a consultant to Coronado Biosciences, which has licensed the patent to clinical commercialization of tumor-primed NK cells.

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