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Resistance to Cytarabine Induces the Up-regulation of NKG2D Ligands and Enhances Natural Killer Cell Lysis of Leukemic Cells[1]

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Abstract

Prolonged treatment of leukemic cells with chemotherapeutic agents frequently results in development of drug resistance. Moreover, selection of drug-resistant cell populations may be associated with changes in malignant properties such as proliferation rate, invasiveness, and immunogenicity. In the present study, the sensitivity of cytarabine (1-β-D-arabinofuranosylcytosine, araC)-resistant and parental human leukemic cell lines (T-lymphoid H9 and acute T-lymphoblastic leukemia Molt-4) to natural killer (NK) cell–mediated killing was investigated. The results obtained demonstrate that araC-resistant H9 and Molt-4 (H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100}) cell lines are more sensitive to NK cell–mediated lysis than their respective parental cell lines. This increased sensitivity was associated with a higher surface expression of ligands for the NK cell–activating receptor NKG2D, notably UL16 binding protein-2 (ULBP-2) and ULBP-3 in H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines. Blocking ULBP-2 and ULBP-3 or NKG2D with monoclonal antibody completely abrogated NK cell lysis. Constitutive phosphorylated extracellular signal–regulated kinase (ERK) but not pAKT was higher in araC-resistant cells than in parental cell lines. Inhibition of ERK using ERK inhibitor PD98059 decreased both ULBP-2/ULBP-3 expression and NK cell cytotoxicity. Furthermore, overexpression of constitutively active ERK in H9 parental cells resulted in increased ULBP-2/ULBP-3 expression and enhanced NK cell lysis. These results demonstrate that increased sensitivity of araC-resistant leukemic cells to NK cell lysis is caused by higher NKG2D ligand expression, resulting from more active ERK signaling pathway.

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Introduction

Nucleoside analogs represent a group of cytotoxic antimetabolites in the treatment of hematological malignancies, solid tumors and viral infections [1–4]. They mimic physiological nucleosides and share their metabolic pathways. Cytarabine (1-β-D-arabinofuranosylcytosine, araC), a deoxycytidine analog, is one of the most important antileukemic drugs currently available for the treatment of acute myeloid leukemia [5,6], relapsed and refractory acute lymphoblastic leukemia [7–9], and large cell lymphoma [10]. Prolonged, *in vitro* and *in vivo*, treatment with araC has, however, resulted in the emergence of drug resistant cells with diminished sensitivity to the drug and ultimately contributing to treatment failures [6,11–13].

Acquired drug resistance of leukemic cells caused by pretreatment with cytostatic drugs influences the sensitivity of leukemic cells toward cytotoxic lymphocytes [14–19]. Whereas some reports show decreased sensitivity of drug-resistant leukemic cells to cellular cytotoxicity [17,18,20,21], Posovszky et al. [19] reported that chemotherapeutic drugs including araC sensitize pre-B acute lymphoblastic leukemia (ALL) cells for CD95- and cytotoxic T-lymphocyte–mediated apoptosis. Recent studies have shown that natural killer (NK) cells display cytolytic activities by engagement of receptors involved in

Abbreviations: AraC, cytarabine; 1-β-D-arabinofuranosylcytosine; E/T, effector-target; ERK, extracellular signal–regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-2, interleukin-2; IMDM, Iscove's modified Dulbecco's medium; MHC-I, major histocompatibility complex class I; MICA/B, MHC-I–related chain A/B; NK, natural killer; NKG2D, natural killer group 2D; ULBP, UL-16 binding protein

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NK cell activation and inhibition [22]. Although NK cells can kill target cells spontaneously without prior stimulation, a delicate balance between signaling through inhibitory [killer immunoglobulin-like receptors (KIR), CD94-NK group 2, member A (NKG2A)] and activating receptors [natural cytotoxic receptors (NCRs-NKp30, NKp44, and NKp46), NK group 2, member D (NKG2D), and DNAX accessory molecule-1 (DNAM-1)] tightly regulates their activation [22].

The relevance of the NKG2D/NKG2D ligand system for the immune surveillance in patient leukemia cells was previously described [23]. Salih et al. [23] reported that leukemia cells from patients variously express major histocompatibility complex class I (MHC-I)–related chain A/B (MICA/B) and UL16 binding protein (ULBP). They also showed that patient leukemia cells were lysed by NK cells in an NKG2D-dependent fashion. The NKG2D receptor is constitutively expressed on the cell surface of human NK cells [24], γδT cells, and $CD8^+$ αβT cells [25]. The proposed role of the NKG2D receptor in innate and adaptive immune responses to cellular and tissue stress is based on the ability of the receptor to stimulate cytotoxic effects of NK cells and T cells against virally infected cells and tumor cells *in vitro* and *in vivo* [26]. Specifically, NKG2D receptor activation can induce target cell lysis and trigger the production of cytokines [27,28] and chemokines [27,29,30], as well as perforin and granzyme involved in cellular lysis [31]. DNAX accessory molecule-1, a coactivating receptor of NK cells, is another surface molecule that has been shown to participate in NK cell activation [32,33]. DNAX accessory molecule-1 is known to be involved not only in NK cell activation but also in cell-cell adhesion [34]. It has been shown that the susceptibility of tumor cells to NK cell–mediated lysis is dependent on the expression level of polio virus receptor (PVR) specifically recognized by DNAM-1 [32,33,35,36].

In this study, the expression of ligands of NK cell–activating and –inhibitory receptors on parental and $H9^rARAC^{100}$ and Molt-4^rARAC^{100} cell lines and their function in NK cell–mediated cytolytic activity were investigated. The possible mechanism involved in the expression pattern of the ligands was also studied.

Materials and Methods

Cell Lines

The human T cell lymphoma H9 and acute T lymphoblastic leukemia Molt-4 cell lines were obtained from the American Type Culture Collection (Rockville, MD; ATCC Nos. HTB-176 and CRL 1582, respectively). The araC-resistant H9 and Molt-4 cell sublines were established by exposing parental cells to increasing concentrations of the drug. The resistant sublines were grown for more than 6 months in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and containing 100 μM araC (designated $H9^rARAC^{100}$ and Molt-4^rARAC^{100}, respectively). All experiments were performed using araC-resistant cells subcultured at 5-day intervals without further addition of drug. All culture media and media supplements were purchased from Seromed (Berlin, Germany). The cells were propagated in IMDM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. The NK cell sensitive erythroleukemic cell line K562 (ATCC No. CCL-243) was maintained in IMDM supplemented with 20% FCS and used as control for NK cell cytotoxicity.

MTT Assay

Cell viability was investigated using the modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium-bromide (MTT) assay as previously described [37]. Briefly, cells were grown in 96-well plates with and without addition of drugs. After the incubation period, MTT reagent was added for 4 hours. Thereafter, 100 μl of sodium dodecyl sulfate (SDS) solution (20% SDS in a 1:1 dimethyformamide/H_2O solution) was added for a further 4 hours. Plates were read on a multiwell scanning spectrophotometer (Tecan, Crailsheim, Germany) at a wavelength of 560 nm and a reference wavelength of 620 nm. Cell viability was determined as the relative reduction of the amount of MTT reduced by cells to its purple formazan derivative, which correlates with the amount of viable cells in relation to cell control.

Polyclonal NK Cells Preparation

Human peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll-Hypaque centrifugation. Freshly isolated peripheral blood mononuclear cells were incubated for 2 hours at 37°C to allow adherence of monocytes to the bottom of the culture flasks. The cell suspension was collected and NK cells were separated according to manufacturer's protocol using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The separated NK cells were treated with 100 U/ml recombinant human interleukin-2 (IL-2; Cell Concepts, Umkirch, Germany) for 5 days. Flow cytometric analysis to determine the purity of NK cells showed that more than 90% of the cells were $CD56^+CD3^-$ (data not shown).

Antibodies, Reagents, and Measurement of Cell Surface Receptors

AraC was obtained from Sigma (Deisenhofen, Germany), the ERK inhibitor PD98059 was from Calbiochem (Darmstadt, Germany), and purified NKG2D was from BD Pharmingen (San Diego, CA). For quantitative analysis of the expression of PVR, MICA/B, ULBP-1, ULBP-2, ULBP-3, and MHC-I, a one-color cytofluorometric analysis (FACScan; Becton Dickinson, Heidelberg, Germany) was carried out. Cells were stained with mouse monoclonal to PVR, ab3142 (Abcam, Cambridge, UK), antihuman MICA/MICB monoclonal antibody (mAb), BAMO1 (Immatics, Tuebingen, Germany), ULBP1-3 (R&D Systems, Wiesbaden, Germany) followed by phycoerythrin (PE)-conjugated goat antimouse IgG second reagent (R&D Systems). Fluorescein isothiocyanate–conjugated mouse monoclonal antihuman HLA class I (Biosource, CA) was used for MHC-I expression measurements.

Natural Killer Cytotoxicity Assay

Natural killer cells were tested for cytolytic activity against indicated target cells using the "aCella-Tox" kit (Cell Technology, Mountain View, CA) as previously described [36]. Briefly, target cells were plated in triplicate (5000 cells per well) in a 96-well white plate (Greiner Bio-One, Frickenhausen, Germany). Effector cells (NK cells) at indicated effector to target (E/T) ratios were added. Spontaneous effector and target cell death was accomplished by including control wells of effector and target cells at numbers corresponding to those of their various E/T ratios. To determine maximum release [total glyceralehyde-3-phosphate dehydrogenase (G3PDH) released], 10 μl of lytic reagent (0.5% NP-40/100 μl sample) was added to the target cells positive control 10 minutes to end of assay incubation. At the end of 4 hours of incubation, 100 μl of 2× enzyme assay reagent was added to each well. Fifty microliters of 1× detection reagent was

immediately added to each well. The plate was read at once in a luminometer (Glomax; Promega, Mannheim, Germany). For the mAb-mediated neutralization experiments, NK cells were first incubated for 1 hour with 50% human serum (to prevent binding of the mAbs to the various Fc receptors expressed on the surface of human NK cells) and washed. Leukemic and NK cells were then incubated initially at room temperature for 20 minutes with 20-μg/ml indicated mAb. End concentration of mAb for the 4-hour assay was 4 μg/ml. Isotype control IgG (Becton Dickinson, San Jose, CA) was used as negative control. Iscove's modified Dulbecco's medium supplemented with 1% heat-inactivated FCS was used as assay medium. The percent cytotoxicity was calculated as follows: [(experimental G3PDH release – spontaneous G3PDH release from effector cells alone – spontaneous G3PDH release from target cells alone) / (maximum G3PDH release from target cells – spontaneous G3PDH release from target cells)] × 100. The spontaneous target cell release was always <20% of maximum release.

Real-time Polymerase Chain Reaction

Total RNA was isolated using TRI reagent (Sigma-Aldrich, Seelze, Germany) according to the manufacturer's protocol. Reverse transcription was carried out with reagents from Applied Biosystems (Foster City, CA) according to the manufacturer's instructions. cDNA was quantified by quantitative real-time polymerase chain reaction (PCR) with the ABI PRISM 7900HT. All reactions were performed using SYBR Green assays according to a standard thermal profile: denaturation at 95°C for 15 seconds, annealing/extension at 60°C for 60 seconds with 40 repeats. Primers for ULBP-2 are as follows: forward 5′-CCC TGG GGA AGA AAC TAA ATG TC-3′; reverse 5′-ACT GAA CTG CCA AGA TCC ACT GCT-3′. Primers for ULBP-3 are as follows: forward 5′-AGA TGC CTG GGG AAA ACA ACT G-3′; reverse 5′-GTA TCC ATC GGC TTC ACA CTC ACA-3′. Primers for β-actin are as follows: forward 5′-CGC GAG AAG ATG ACC CAG AT-3′; reverse 5′-CAG AGG CGT ACA GGG ATA GCA-3′. All the samples were performed at least in duplicates. Threshold levels and baseline were optimized. Relative quantification was determined with the SDS2.1 software (Applied Biosystems) provided with the ABI PRISM 7900HT ($\Delta\Delta C_t$ method). Normalization was obtained by using β-actin as endogenous control and the parental cell culture as calibration sample in comparison to the araC-resistant cell cultures. The results are presented as fold increase.

Western Blot

Western blot analysis was performed as previously described [38]. Briefly, cell lysates were subjected to SDS-PAGE before transferring to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using the Mini-Protean II System (Bio-Rad, Munich, Germany). After transfer, blots were blocked in Tris-buffered saline blocking buffer containing 3% bovine serum albumin for 1 hour at room temperature to saturate the nonspecific protein-binding sites on the nitrocellulose membrane. The following primary rabbit polyclonal Abs were used: ERK, anti–phospho-ERK1/2, AKT, anti–phospho-AKT (ser 473; Cell Signaling, Beverly, MA). Mouse polyclonal β-actin Ab was from Sigma-Aldrich (Munich, Germany). The blots were incubated overnight with the primary Ab diluted in Tris-buffered saline at 4°C with gentle agitation. After a 1-hour incubation period with peroxidase-conjugated secondary Ab at room temperature, visualization was performed by enhanced chemiluminescence using a commercially available kit (Amersham, Liverpool, UK).

Inhibition of ERK Activation

Inhibition of ERK activation was performed by pretreating both parental and araC-resistant cells for 48 hours with 20 μM PD98059.

Transfection and Plasmid

The constitutively active ERK 2 expression plasmid was kindly provided by Professor Stefanie Dimmeler (Frankfurt, Germany). Plasmids included (pcDNA3.1; empty vector) as a control and constitutively active ERK (pcDNA3.1 ERK2). A total of 2×10^6 H9 cells were transfected with 2 μg of plasmid using Amaxa Nucleofection Technology (Cologne, Germany). Transfection efficiency was approximately 58% as determined using green fluorescent protein, and maximal levels of protein expression were observed between 72 and 96 hours.

Measurement of Interferon-γ Production

A total of 2×10^4 leukemic cells (H9, H9^{r}ARAC^{100}, Molt-4, and Molt-4^{r}ARAC^{100}) were cocultured with 2×10^5 NK cells for 24 hours. Natural killer cells alone or leukemic cells were used as control. Supernatants were collected and tested for production of interferon-γ (IFN-γ). The amounts of IFN-γ were determined using the Quantikine Human IFN-γ ELISA kit (R&D Systems) according to the manufacturer's protocol.

Statistics

Values presented are the mean ± SD of at least three experiments. Comparisons between two groups were performed using Student's *t* test. *P* values <.05 were considered to be significant.

Results

Viability of Leukemic Cells on araC Treatment

MTT assay was performed to determine the viability of the leukemic cell lines used on treatment with araC. Cytotoxic effects of araC, expressed as concentrations inhibiting 50% of cell growth (IC_{50}), were decreased in araC-resistant cells when compared with parental cells. Resistance indexes (RIs; ratio of IC_{50} in H9^{r}ARAC^{100} and H9 cells as well as in Molt-4^{r}ARAC^{100} and Molt-4 cells) of araC for H9 and Molt-4 cells were 2.2×10^4 and 4.3×10^4, respectively (Table 1).

Cytotoxic Activity of NK Cells Against Leukemic Cell Lines

The cytolytic activities of IL-2–activated NK cells against parental as well as H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines were determined. The results show that NK cells effectively kill parental H9 and Molt-4 cell lines. Both araC-resistant cell lines showed higher sensitivity to NK cell lysis than parental cell lines (11% and 55%

Table 1. Cytotoxic Effects of araC in Molt-4, Molt-4^{r}ARAC^{100}, H9, and H9^{r}ARAC^{100} Cells.

Cells	IC_{50} (μM)*		RI†
	Without PD98059	With PD98059	
Molt-4	0.029 ± 0.0018	0.024 ± 0.0016	4.3×10^4
Molt-4^{r}ARAC^{100}	1256 ± 234	1450 ± 195	
H9	0.034 ± 0.0047	0.031 ± 0.0051	2.2×10^4
H9^{r}ARAC^{100}	756.8 ± 53.7	695.6 ± 78.9	

PD98059 was added at a concentration of 20 μM.
*Results represent mean values ± SD of three different experiments.
†RI (ratio IC_{50} resistant, IC_{50} parental cell lines without PD98059).

at E/T 1:1 and 4:1, respectively, for H9^{r}ARAC^{100} *vs* 6% and 38% at E/T 1:1 and 4:1, respectively, for H9; 29% and 70% at E/T 1:1 and 4:1, respectively, for Molt-4^{r}ARAC^{100} *vs* 4% and 31% at E/T 1:1 and 4:1, respectively, for Molt-4; Figure 1, *A* and *B*). To exclude the direct effects of araC on leukemic cell sensitivity to NK cells, resistant cells used for the experiment were cultured for at least three subcultures without the drug. Similar results were obtained with the cells cultured up to 10 passages without the drug (not shown). Natural killer cell lysis of K562 cell line was used as positive control, and it resulted to approximately 90% lysis (E/T 4:1; not shown). The cytolytic activities of freshly isolated NK cells against parental as well as H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines were also tested. Both araC-resistant cell lines also showed higher sensitivity to NK cell lysis than their parental counterparts. Lysis of leukemic cells was, however, detectable only at higher E/T ratios (32% and 62% at E/T 10:1 and 20:1, respectively, for H9^{r}ARAC^{100} *vs* 20% and 45% at E/T 10:1 and 20:1, respectively, for H9; 47% and 58% at E/T 10:1 and 20:1, respectively, for Molt-4^{r}ARAC^{100} *vs* 30% and 41% at E/T 10:1 and 20:1, respectively, for Molt-4; Figure 1, *C* and *D*). The production of IFN-γ by NK cells was also measured upon coculture of NK cells with parental as well as H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines. Cocultures of NK cells with parental H9 and Molt-4 cells resulted in 320 ± 25 and 57 ± 12 pg/ml IFN-γ, respectively, for NK/H9 and NK/Molt-4 cocultures. The IFN-γ production was increased 2.25- and 1.7-fold, respectively, in NK/H9^{r}ARAC^{100} and NK/Molt-4^{r}ARAC^{100} cocultures. IL-2–activated NK cells were used for further cytotoxic experiments because low E/T ratios are required.

Expression of NK Cell–Activating and –Inhibitory Ligands on Surface of Leukemic Cells

The expression pattern of ligands for the NK cell–activating/inhibitory receptor on the surface of both parental and araC-resistant cell lines was investigated to show whether it correlates with the lysis of leukemic cells by NK cells. Results obtained show that increased NK cell lysis of leukemic cells was associated with a corresponding increase in the cell surface expression of ULBP-2, ULBP-3, and PVR in H9^{r}ARAC^{100} (relative fluorescent units: 380 *vs* 217 for ULBP-2, 122 *vs* 50 for ULBP-3, and 43 *vs* 27 for PVR; Figure 2*A*). In Molt-4^{r}ARAC^{100} cells, ULBP-2 and ULBP-3 cell surface expression were highly increased, whereas no change was observed for PVR (relative fluorescent units: 82 *vs* 29 for ULBP-2, 31 *vs* 6 for ULBP-3, and 10 *vs* 8 for PVR; Figure 2*A*). There was no significant change in MHC-I expression level in both parental and araC-resistant cell lines. Both parental and araC-resistant leukemic cell lines did not express MICA/B or ULBP-1 except for Molt-4, which showed minimal expression of ULBP-1 (not shown). The mRNA expression of araC-resistant and parental cell lines used for this study was also evaluated using real-time PCR. As shown in Figure 2*B*, an increased ULBP-2 and ULBP-3 mRNA expression was observed in araC-resistant cell lines (approximately two-fold ULBP-2, six-fold ULBP-3 increase for H9^{r}ARAC^{100} and six-fold ULBP-2, two-fold ULBP-3 for Molt-4^{r}ARAC^{100}) in comparison to their respective parental cell lines. This indicates that araC modifies the NK cell–activating receptor ligand expression at the transcriptional level. The effect of this modification seems to be maintained because several passaging of leukemic cells continued to display this effect (not shown).

NK Cell Recognition of Leukemic Cell Lines Through NKG2D

In an attempt to understand a possible mechanism of the increased NK cell lysis of resistant leukemic cells as well as the functionality of NKG2D ligands, mAb masking experiments were performed. As shown in Figure 3, blocking PVR on H9 and H9^{r}ARAC^{100} cells did not show significant decrease in NK cell lysis (36% *vs* 33% and 58% *vs* 50%, respectively for H9 and H9^{r}ARAC^{100}). However,

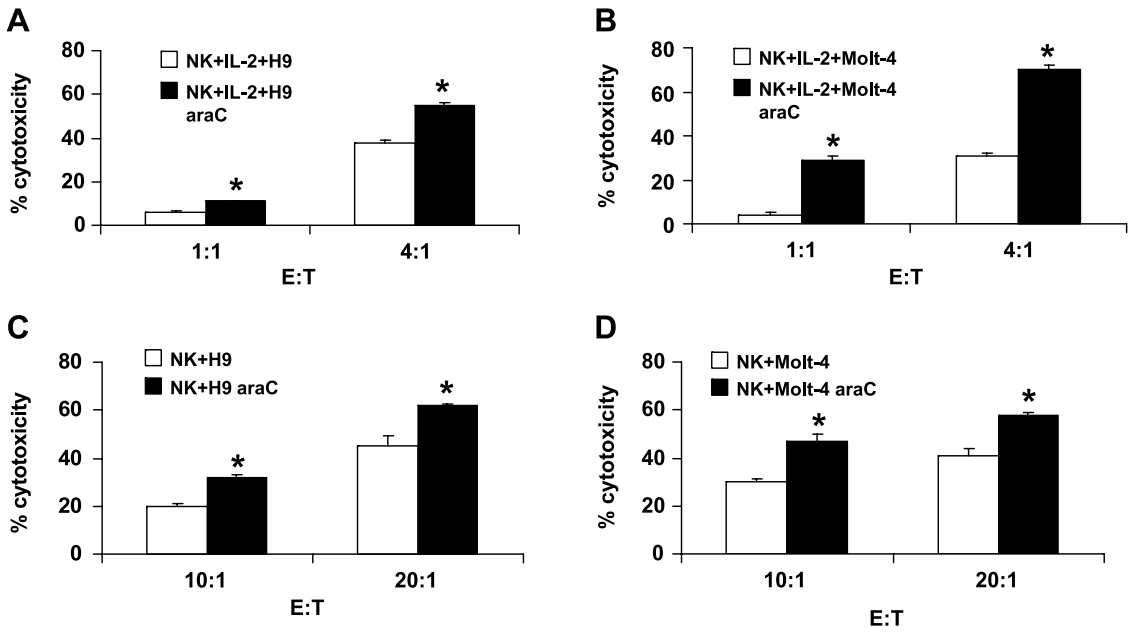

Figure 1. Natural killer cell cytotoxicity of parental and araC-resistant leukemic cells. Killing of H9 and H9^{r}ARAC^{100} (H9 araC) as well as Molt-4 and Molt-4^{r}ARAC^{100} (Molt-4 araC) cell lines was assessed by G3PDH release using acella-Tox kit. Five thousand target cells were coincubated with IL-2–activated NK cells (A and B) or freshly isolated NK cells (C and D) at the indicated E/T ratios for 4 hours at 37°C. The results are mean ± SD of three independent experiments. *$P < .05$ compared with parental control.

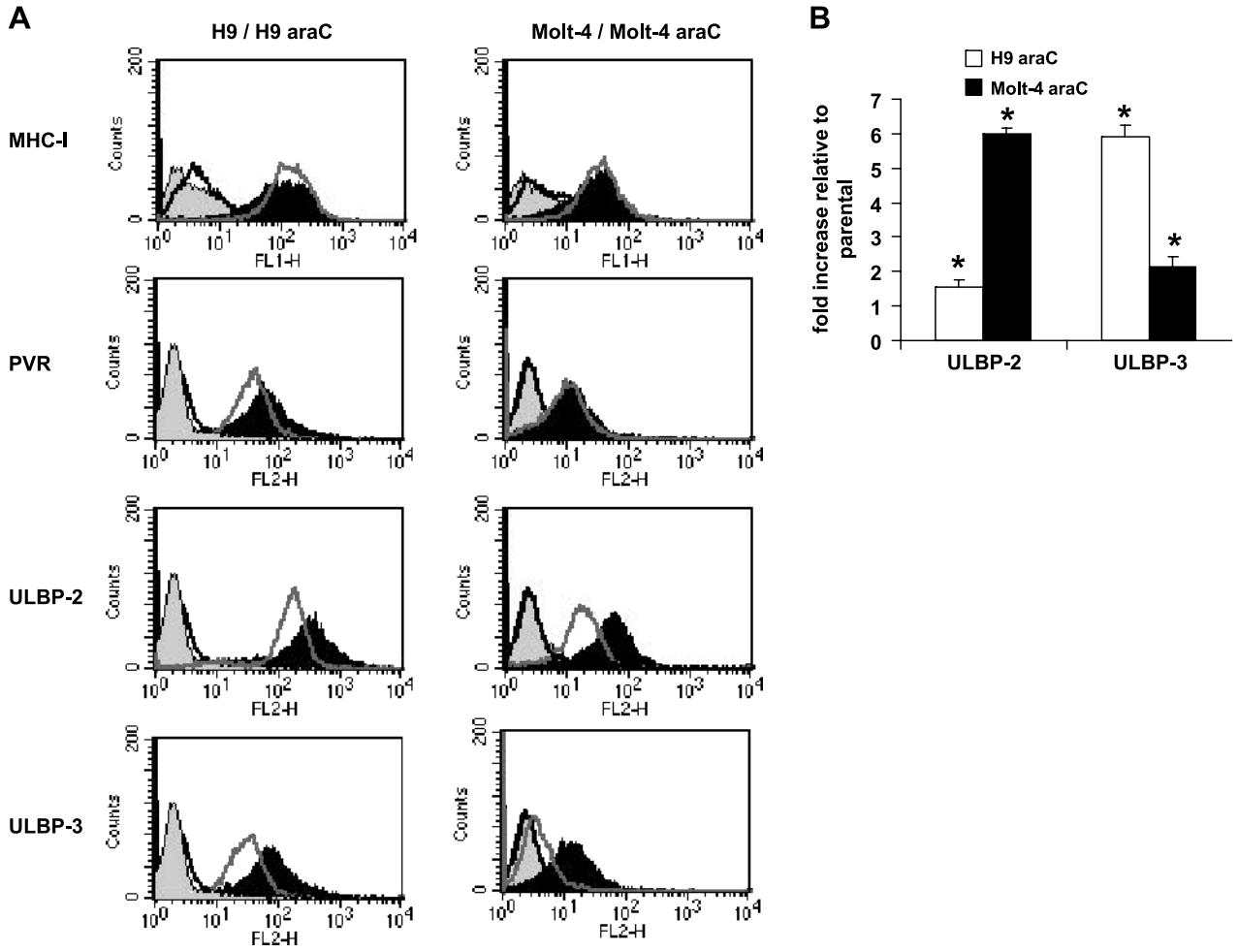

Figure 2. Expression of ligands for NK cell receptors in leukemic cells. (A) Flow cytometric analysis for the expression of ligands in H9 and H9^{r}ARAC^{100} cells and Molt-4 and Molt-4^{r}ARAC^{100} cells. Black line shows isotype control staining, and black-filled histogram shows indicated antibody staining for both H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines. Gray-filled histograms shows isotype control staining, and gray line shows indicated antibody staining for both H9 and Molt-4 cell lines. One representative of at least three separate experiments is shown. (B) Real-time reverse transcription–PCR for the mRNA expression levels of ULBP-2 and ULBP-3 transcripts. Data are expressed as mRNA fold increase in H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cells relative to parental H9 and Molt-4 cells. Histograms are representative of three different experiments. Each experiment was run in duplicate; error bars indicate ±SD. *$P < .05$ compared with parental control.

blocking ULBP-2 or ULBP-3 alone showed a strong inhibition of NK cell lysis (36% *vs* 3% ULBP-2 and 4% ULBP-3, 58% *vs* 4% ULBP-2 and 15% ULBP-3, respectively, for H9 and H9^{r}ARAC^{100}). In both H9 and H9^{r}ARAC^{100} cell lines, the expression level of ULBP-2 and ULBP-3 was approximately eight- and two-fold higher, respectively, than that of PVR. Combined blocking of ULBP-2 and ULBP-3 on H9^{r}ARAC^{100} cells resulted in total abrogation of NK cell lysis. A 100% inhibition of NK cell lysis was also observed on blocking NKG2D on NK cells (Figure 3). Similar results were obtained using Molt-4 and Molt-4^{r}ARAC^{100} cell lines (not shown). The results suggest that NK cell activation through NKG2D receptor–ligand binding is the possible mechanism involved in the increased lysis of H9^{r}ARAC^{100} and Molt-4^{r}ARAC^{100} cell lines.

Possible Mechanism of Increased Ligand Expression in araC-Resistant Leukemic Cells

Extracellular signal–regulated kinase and AKT may influence sensitivity of leukemic cells to araC [39–41]. Moreover, both ERK and

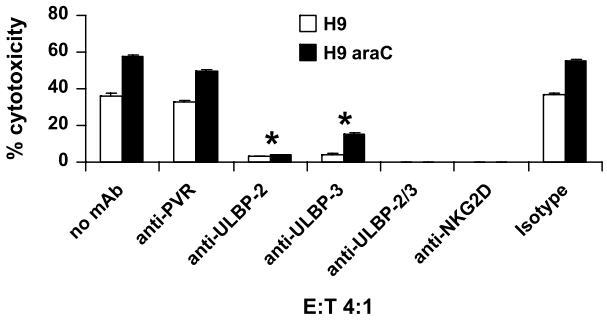

Figure 3. Natural killer cells recognize leukemic cells through NKG2D receptor. Five thousand H9 and H9^{r}ARAC^{100} cells were coincubated with IL-2–activated NK cells at an E/T ratio of 4:1 for 4 hours at 37°C either in the absence or in the presence of mAb. IgG isotype control was used as negative control. The results are mean ± SD of three independent experiments. *$P < .05$ compared with cells not blocked with mAb.

AKT were shown to be involved in the regulation of expression of NKG2D ligands [42,43]. For these reasons, both ERK and AKT signaling pathways were studied for their constitutive activation status in both parental and araC-resistant cells. Western blot analysis using parental H9 as well as H9^{r}ARAC^{100} cells showed a stronger constitutive phosphorylated ERK but not AKT in H9^{r}ARAC^{100} cells (Figure 4*A*). To verify the role of ERK signaling pathway in NKG2D ligand expression, flow cytometric analysis and cytotoxicity assay were performed on ERK inhibition of parental H9 and H9^{r}ARAC^{100} cells using 20 μM PD98059. Treatment of both parental and araC-resistant leukemic cell lines with PD98059 shows only minimal effect on cell viability (Table 1). It, however, suppressed their ERK activation status, decreased ULBP-2 (relative expression from 182 to 152 and 266 to 102 for H9 and H9^{r}ARAC^{100}, respectively) and ULBP-3 (relative expression from 24 to 14 and 60 to 49 for H9 and H9^{r}ARAC^{100}, respectively) surface expression and impaired NK cell lysis (from 50% to 39% and 68% to 43% for H9 and H9^{r}ARAC^{100}, respectively; Figure 4, *B–D*). These results were further confirmed by transfecting parental H9 cells with constitutively active ERK (Figure 5). Transfection of parental H9 cells with constitutively active ERK resulted to 1.9- and 2.1-fold higher ULBP-2 and ULBP-3 ligand expression, respectively, when compared with parental H9 cells (Figure 5*B*). It also enhanced NK cell lysis by 1.52-fold compared with parental H9 cells (Figure 5*C*). Furthermore, blocking ULBP-2 and ULBP-3 with mAb inhibited NK cell lysis (Figure 5*D*). Taken together, these results demonstrate that increased sensitivity of araC-resistant leukemic cells to NK cell lysis is caused by higher NKG2D ligand expression, resulting from more active ERK but not AKT signaling pathway.

Discussion

Numerous experimental studies demonstrated that drug exposure may induce not only resistance but also change other properties of tumor cells which may be related to tumor growth, invasiveness, and immunogenicity [36,44–47]. The present study shows that resistance of T-cell leukemia cell lines to araC is associated with increased sensitivity to NK cell–mediated lysis. These effects were not caused by direct activity of araC on cell metabolism but rather by selection of cell population with altered susceptibility to NK cells because the resistant cells cultured for at least three passages in a medium without the drug were lysed by NK cells to a greater extent than the parental cell population.

Selection of chemotherapy-resistant tumor cells may be associated with increased immunogenicity. Although increased immunogenicity of malignant cells resistant to chemotherapeutic agents has been reported previously [19,36], mechanisms responsible for such effects

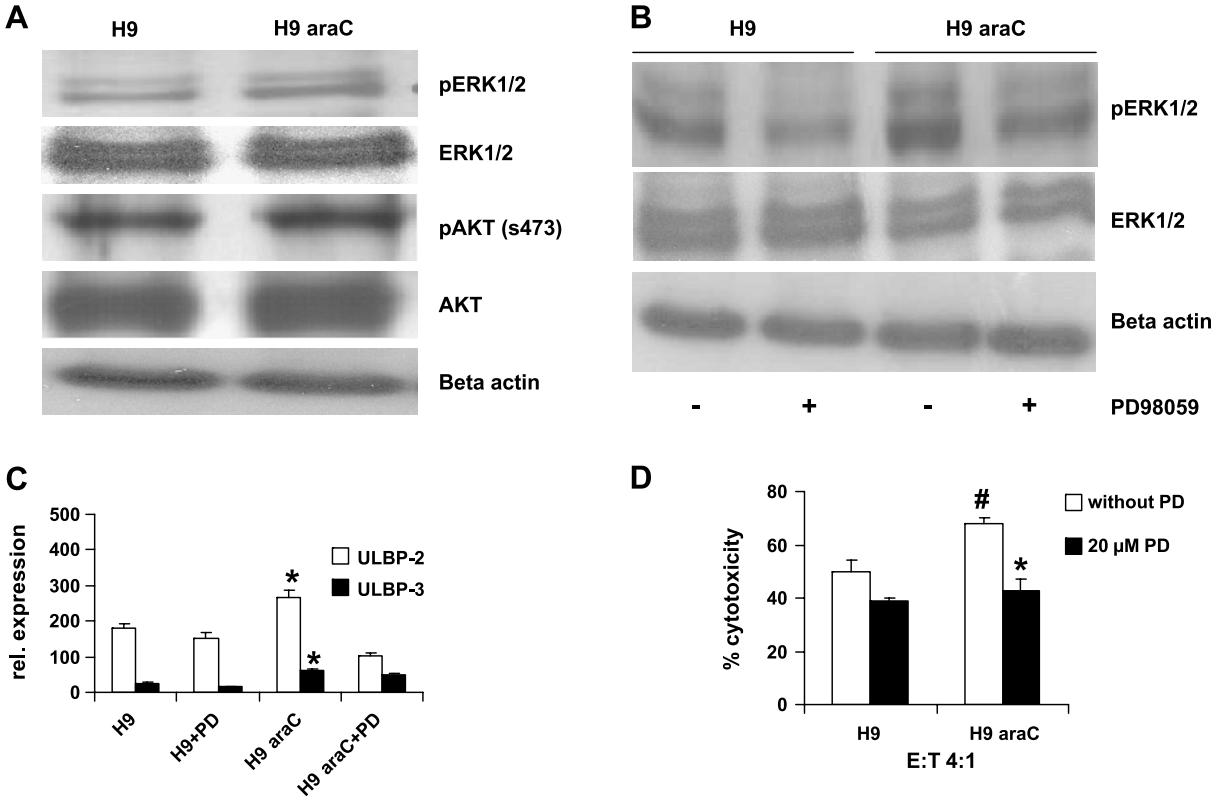

Figure 4. Mechanism of increased ligand expression in araC-resistant cells. (A) The same amount of cell extracts prepared from the same passage of H9 and H9^{r}ARAC^{100} cell cultures was used for Western blot analyses. Constitutive AKT and ERK activation was assessed using antibodies that recognize AKT phosphorylated at ser-473 and ERK1/2 phosphorylated at Thr202/Tyr204. (B) Western blot analysis of H9 and H9^{r}ARAC^{100} cells after specific inhibition of ERK activation using PD98059. Data are representative of at least three experiments. (C) Flow cytometric analysis for ULBP-2 and ULBP-3 expression in H9 and H9^{r}ARAC^{100} cells after a 48-hour treatment with PD98059 (PD). Results are representative of three different experiments. Error bars indicate ±SD, $^*P < .05$ compared with parental H9 cell line. (D) IL-2–activated NK cell cytotoxicty of H9 and H9^{r}ARAC^{100} cells after a 48-hour treatment with PD98059. Results are representative of three different experiments. Error bars indicate ±SD, $^*P < .05$ compared with H9^{r}ARAC^{100} cell line without PD. $^{\#}P < .05$ compared with parental H9 cell line without PD.

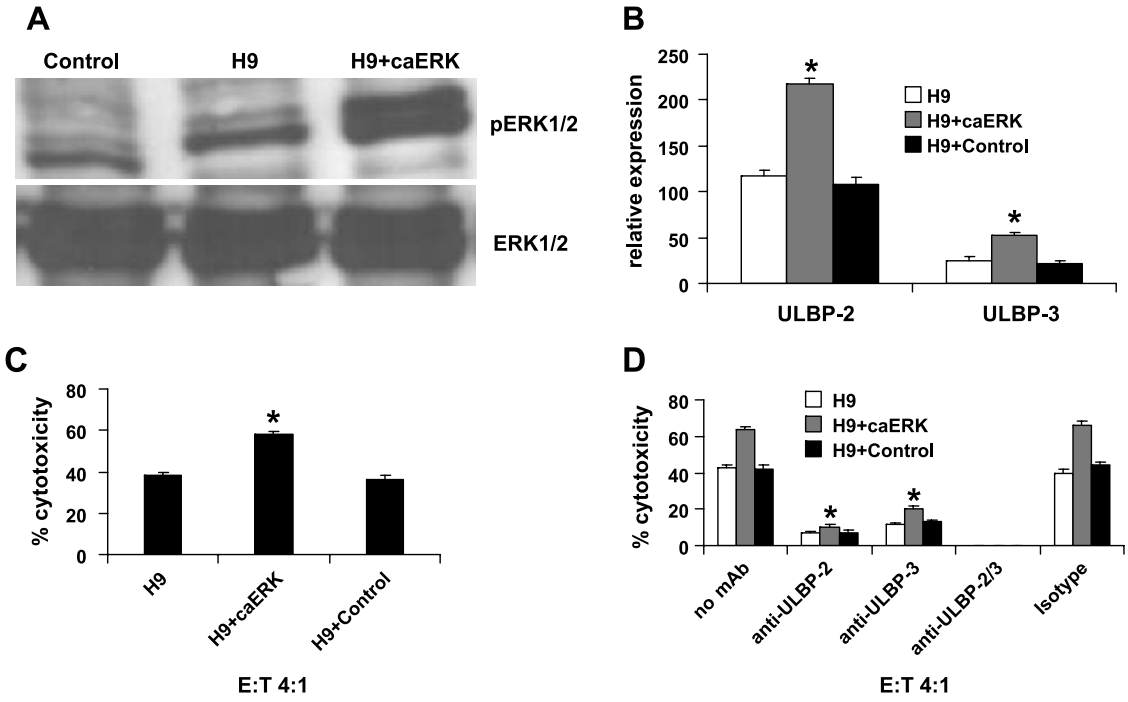

Figure 5. ERK activation is responsible for increased NKG2D ligand expression. (A) ERK activation was assessed by Western blot analysis of constitutively active ERK (caERK)-transfected H9. Empty vector–transfected H9 cells were used as control. (B) Flow cytometric analysis for the expression of NKG2D ligands after transfection of H9 cells with caERK. Results are representative of three different experiments. Error bars indicate ±SD, $^*P < .05$ compared with parental H9 cells (C) NK cell cytotoxicity experiments were performed after transfection of H9 cells with caERK. Results are representative of three different experiments. Error bars indicate ±SD, $^*P < .05$ compared with parental H9 cells. (D) mAb blocking experiments were performed to demonstrate the role of ULBP-2 and ULBP-3 in NK cell lysis after transfection of H9 cells with caERK. Results are representative of three different experiments. Error bars indicate ±SD, $^*P < .05$ compared with cells not blocked with mAb.

remain poorly understood. This work focuses on the role of specific ligands for NK cell–activating receptors in the susceptibility of parental and araC-resistant leukemic cells to NK cell lysis. H9 and Molt-4 cells express NKG2D ligands (particularly ULBP-2 and ULBP-3) in addition to PVR (ligand for NK cell–coactivating receptor DNAM-1), whereas they do not express other NKG2D ligands including MICA and MICB. This is in concordance with the hypothesis by Pende et al. [48] stating that most T-cell leukemia cell lines are characterized by a $MICA^-ULBP^+$ phenotype. The results, with particular reference to the blocking experiments, show that increased expression of ULBP-2 and ULBP-3 rendered araC-resistant leukemic cell lines to become more sensitive to NK cell lysis. Blocking PVR with mAb could not inhibit NK cell lysis, suggesting that DNAM-1 is not involved in NK cell lysis of H9 and Molt-4 cell lines. The increased NK cell lysis of $H9^rARAC^{100}$ and Molt-4^rARAC^{100} cells could still be observed despite expression of MHC-I molecules on their surface. These findings suggest that ULBP-NKG2D interaction is a major determinant for susceptibility of H9 and Molt-4 cell lines to NK cell lysis.

The expression of NK cell–activating receptor ligands, especially NKG2D ligands, has been shown to be associated with malignant transformation, whereas they are generally only transiently expressed on healthy tissues [49]. Recent experiments demonstrated that NKG2D/NKG2D ligand system stimulates immune surveillance of tumors [50–52]. The capacity of the NKG2D ligand–expressing tumor cell lines to stimulate tumor immunity *in vivo* was critically dependent on the expression levels of NKG2D ligands on the tumor cell surface. In previous studies, NKG2D ligand surface expression led to increased susceptibility of malignant or virus-infected cells to NK cell– or T cell–mediated lysis [27,28]. Because NK cell activity is guided by a balance of activating and inhibitory signals, and an enhanced NKG2D ligand expression is able to trigger NK cells overcoming inhibitory signals by MHC-I molecules [25,27], even modest changes in NKG2D ligand expression may critically influence NK cell cytotoxic potential. The significance of NKG2D signaling in protection against infection and tumorigenesis is highlighted by the development of mechanisms by viruses and tumor cells to evade detection by this system. Independent reports by Oppenheim et al. [53], Wiemann et al. [54], and Coudert et al. [55], described the down-modulation and altered function of NKG2D in immune effector cells after chronic exposure to its ligands on tumor cells. Therefore, in addition to being involved in increased protection against tumor progression, enhanced expression of NKG2D ligands on surface of araC-resistant cells may also contribute to the evasion of leukemic cells from NK cell reactions during chronic exposure of NKG2D on NK cell surface to NKG2D ligands expressed on the surface of leukemic cells.

Several mechanisms of resistance to araC, such as increased inactivation of araC by cytidine deaminase, decreased intracellular permeation, decreased cellular activation by deoxycytidine kinase, increased degradation of araC nucleotides by 5′-nucleotidase, imbalance of cellular deoxynucleotide pools, increased capability of repair of damaged DNA, and decreased expression of human equilibrative nucleoside transporter 1 (hENT1) involved in the transport of araC

across the cell membrane, mutations of *hENT1*, and deoxycytidine kinase genes have been reported [11–13,56–64]. Moreover, cell regulatory pathways such as ERK or AKT may influence the sensitivity of leukemic cells to araC [39–41]. Treatment of leukemic cells with inhibitors of AKT or ERK pathways was shown to increase their sensitivity to araC treatment [39–41]. Experiments performed in this study show both AKT and ERK to be constitutively activated in H9 cells, with increased activation of ERK but not AKT in resistant cells relative to their parental counterparts. Treatment of leukemic cells with pharmacological inhibitor of ERK showed no toxicity to the cells and did not significantly influence activity of araC in resistant cells. However, NKG2D ligand expression in parental and araC-resistant leukemic cells and their corresponding lysis by NK cells were suppressed. These findings demonstrate that ERK activation is not a resistance mechanism in Molt-4 or H9 cells but is involved in the sensitivity of resistant cells to NK cell lysis.

Extracellular signal–regulated kinase was also shown to increase the expression of NKG2D ligands and the sensitivity to NK cell–mediated lysis of transformed cells derived from solid tumors and leukemia [42]. In fact, Borchers et al. [42] reported the involvement of ERK signaling in the induction of surface expression of NKG2D ligands in human tumor cells after H_2O_2-induced oxidative stress. The results presented in this study demonstrate that ERK activation is associated with increased ULBP-2 and ULBP-3 expression resulting in enhanced sensitivity of araC-resistant leukemic cells to NK cell lysis. The increased ULBP-2/ULBP-3 ligand expression in constitutively active ERK-transfected H9 cells and the corresponding enhanced NK cell lysis provide direct evidence for the involvement of ERK activation in NKG2D ligand expression in leukemic cells.

In conclusion, this report shows for the first time that T-cell leukemia cell lines that received araC treatment and became resistant are more sensitive to NK cell attack than their parental counterparts. It is possible that part of the efficacy of some chemotherapies and radiotherapies, most of which activate the DNA damage response [65,66], is due to the induction of NKG2D ligands, which, consequently, enhances sensitivity of the cell to the immune system. The present data suggest that development of resistance to chemotherapeutic drugs may be associated with increased immunogenicity of tumor cells. Because such changes persist after cessation of treatment, it is of interest to show whether emergence of chemotherapy-resistant tumor clones with increased sensitivity to NK cell lysis also appear in patients who become refractory to common treatments with araC or other chemotherapeutic agents. The pathway leading to the up-regulation of NKG2D ligands looks promising in the search of targets for design of therapeutic agents to enhance the immunogenicity of transformed cells while reducing overall toxicity.

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