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ORIGINAL ARTICLE

Relevance of target cell-induced apoptosis as mechanism of resistance against natural killer cells

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Abstract Natural killer (NK) cells contribute to the graft-versus-leukemia effect after allogeneic stem cell transplantation. However, the efficacy of NK cell-mediated tumor cell lysis is limited due to target cell resistance, and target cell-induced apoptosis (TiA) was proposed to contribute to differences in susceptibility to NK cells. Here we analyzed the effects of target cells on the apoptosis of cytokine-activated NK cells in vitro. We found no association of target cell susceptibility and TiA of NK cells in an array of human and murine target-effector cell combinations. Incubation of NK cells with caspase inhibitors blocked TiA incompletely, indicating that TiA is partly based on caspase-independent mechanisms. Modulating NK cell susceptibility against TiA by caspase inhibition did not influence cytotoxic efficacy. Furthermore, we found cytotoxic potential of NK cells to be markedly decreased following first target cell contact. Exhaustion of NK cell activity by first target cell contact was, however, not mediated by TiA. In addition, we found no relevant TiA by lymphoma cell lines against activated murine NK cells. We conclude that TiA represents only a minor factor of target cell resistance against NK cell-mediated cytolysis.

Keywords Human · Murine · NK cells · Apoptosis · Cytotoxicity

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Introduction

Natural killer (NK) cells recognize and destroy certain tumor cells without prior immunization. This phenomenon has been recognized in numerous in vivo and in vitro systems in man and mice [1]. The clinical significance of NK cell-mediated cytotoxicity for tumor eradication has been demonstrated after haploidentical allogeneic hematopoietic stem cell transplantation [2, 3]. However, some target cells escape the immunosurveillance exerted by NK cells [2–5].

In principle, three types of target cell resistance may be distinguished: (1) failure of target cell recognition (afferent deficit); (2) failure of NK cells to destroy a recognized target (efferent deficit); and (3) apoptosis of NK cells induced by target cells (counter attack). The relevance of deficient target cell recognition and inefficient target cell lysis has been described and several mechanisms leading to target cell resistance could be clarified [6–12]. The counter attack mechanism has been addressed for the cytolytic efficacy of αβ T cells, γδ T cells, and interleukin (IL)-2 activated NK cells, yet, yielding contradictory results [13–18].

Initial studies of tumor-induced apoptosis (TiA) in human IL-2-activated NK cells showed involvement of FcγRIII (CD16) [19, 20] and CD2 [21]. Those findings suggested a role of TiA in antibody-dependent cellular cytotoxicity (ADCC), and that TiA is similar to activation-induced cell death of T cells. Furthermore, there is evidence that NK cells secretion of granzyme B is engaged in NK cells apoptosis upon activation [22]. Furthermore, it was shown that TiA of activated NK cells occured in the context of stimulatory natural cytotoxicity receptor (NCR) engagement [23]. Thus, TiA may play a role not only for ADCC but also for direct cytotoxicity of NK cells against

malignant tumor cells. TiA triggered by NCR stimulation depended on “autocrine” Fas/Fas-ligand signaling and consecutive caspase-3 involvement. Caspase inhibition in γδ T cells prevented Fas-induced apoptosis, and in consequence, γδ T cells maintained cytotoxic efficacy when repeatedly challenged with lymphoma cells [24].

We, here, addressed the induction of apoptosis in lymphokine-activated NK cells (LAK) by the different origin of malignant target cells and assessed cytotoxic efficacy and TiA of NK cells in parallel. In a second step, we looked for strategies to shield NK cells against target cell-induced apoptosis.

Material and methods

Cell culture

Human K562 (derived from CML blast crisis), ML2 (AML M4 origin) and Jurkat (derived from T-ALL) cell lines and murine A20 (origin Balb/c mouse with MHC: H-2d), YAC-1 (derived from A/Sn mouse with MHC: H-2a), and WEHI-3 (Balb/c origin) cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium with 25 mM HEPES and GlutaMAX I (Gibco-BRL, Karlsruhe, Germany) containing 10% heat inactivated fetal calf serum (FCS, Gibco-BRL) supplemented with penicillin (Sigma, Steinheim, Germany) and streptomycin (Biochrom, Berlin, Germany; complete RPMI). The cytotoxic human NK cell line NK-92, a generous gift from T. Tonn (Institute for Transfusion Medicine and Immunohematology, Red Cross Blood Donor Service Baden-Wuerttemberg-Hessen, Frankfurt/Main, Germany), was cultured in X-vivo medium (BioWhittaker, Apen, Germany) containing 5% human, CMV negative, AB plasma supplemented with 100 U/ml IL-2 (Cell Concepts, Umkirch, Germany).

NK cell enrichment and immunophenotyping

Human NK cells were obtained by positive enrichment with immunomagnetic beads against CD56 (Miltenyi, Bergisch Gladbach, Germany) from mononuclear cells (MNC) of buffy coat preparations. For immunophenotyping, the following fluorochrome-conjugated monoclonal antibodies were used: CD3-FITC (clone SK7), CD4-PE (clone SK3), CD8-FITC (clone SK1), CD16-FITC (clone NKP15), and CD56-PE (clone NCAM16.2; all Becton Dickinson, Heidelberg, Germany). Purity of CD56 positive NK cell preparations was always >90% with a fraction of CD3 positive cells <5%. To obtain lymphokine-activated killer cells, the CD56+ NK cells were exposed to 500 U/ml IL-2 and 100 U/ml IL-12 (cell concepts) for 18 h. The stimulation conditions were established previously to obtain maximally activated effector cells [25].

Mice were purchased from Charles River Laboratories, Inc. (Wilmington, USA) and maintained at the central animal facility at specific pathogen-free conditions. All animal protocols have been approved by the institutional animal protection committee. Murine NK cells were enriched from splenic MNC of C57BL/6 mice (MHC: H-2b) by immunomagnetic DX5 positive selection (Miltenyi). The purity of DX5 positive NK cells used for experiments was always >90%. To obtain maximally activated effector cells, the stimulation conditions were established in preliminary experiments (data not shown). Here, freshly isolated NK cells were activated for 48 h with IL-2 (1,000 U/ml), IL-7 (40 ng/ml), IL-12 (10 ng/ml), and/or IL-18 (100 ng/ml) as indicated.

Inhibition of apoptosis

For inhibition of apoptosis, Jurkat cells and NK cells were cultured for 1 hour at 37°C and 5% CO_2 as indicated with or without 50 or 200 µM Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (ZVAD, Bachem, Weil, Germany) and 800 µM Ac-Asp-Glu-Val-Asp-aldehyde (DEVD, Bachem). Washing cells was followed by incubation with 1 µM daunorubicin (Daunoblastin®, Pfizer, Karlsruhe, Germany) or coincubation of NK cells with target cells. To measure apoptosis by annexin V/propidium iodide (PI) staining, cells were pelleted and resuspended in staining buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; and 5 mM $CaCl_2$), and PI was conducted at a final concentration of 10 µM, containing 20 µl of annexinV-Fluos (Roche Diagnostics, Mannheim, Germany) in each milliliter buffer and subsequently, analyzed on a FACScan.

Cytotoxicity assay

Flow cytometric analysis of NK cell-mediated cytotoxicity and simultaneous measurement of NK cell necrosis were performed as previously described [26]. Briefly, target cells were stained with 0.1 µl/ml of 3 mM 3,3-dioctadecylox acarbocyanine perchlorate (DiO; Sigma, Munich, Germany) dissolved in dimethyl sulfoxide (Sigma) for 20 min at 37°C. Effector cells were incubated with or without caspase inhibitors as indicated, and thereafter, cells were washed with complete medium. Appropriate dilutions of effector cells to obtain indicated effector to target (E:T) ratio were cocultured with stained target cells for 4 h at 37°C and 5% CO_2. For spontaneous cell death, stained target cells were cultured in complete RPMI. Samples were implemented as triplicates. Cells were analyzed on a flow cytometer (FACScan, Becton Dickinson) identifying DiO stained target cells, as well as PI positive dead cells by green and red

fluorescence, respectively. The specific lysis of target cells and of NK cells was calculated using the following equation: specific lysis (%) $=$ (experimental, % $-$ spontaneous %) / (100 $-$ spontaneous %) $\times$ 100.

Apoptosis assay

For flow cytometric analysis of NK cell-mediated apoptosis, target cells of K562, ML2, and Jurkat cell lines were stained with PKH-26 (Sigma). Effector cells were preincubated with or without caspase inhibitors as indicated and thereafter, washed with complete medium. Appropriate cell dilutions to obtain indicated effector to target (E:T) ratios were cocultured with stained target cells for 4 h at 37°C and 5% CO_2. For spontaneous apoptosis, stained target cells were cultured in complete RPMI. Apoptosis was measured by annexinV binding (see above) [27, 28], separating target cells by PKH-26 specific red fluorescence. The specific apoptosis of target cells and LAK were calculated using the following equation: specific apoptosis (%) $=$ (apoptosis of sample % $-$ spontaneous apoptosis %) / (100 $-$ spontaneous apoptosis %) $\times$ 100.

Cold target inhibition assay

Consumption of NK cells cytotoxic activity by contact with target cells was determined in a cold target inhibition assay as previously described [29]. Briefly, NK-92 effector cells were incubated with or without target cells at an effector to target ratio of 1:1 for 4 h at 37°C and 5% of CO_2. Cytotoxicity assays were performed as described above. Inhibition of K562 lysis by pre/coincubation with target cells were calculated using the following equation: inhibition (%) $=$ (without pre $-$ coincubation % $-$ with pre$-$ coincubation %) / without pre $-$ coincubation % $\times$ 100.

Statistical analysis

Differences between cohorts of samples with and without experimental intervention were tested using the Mann–Whitney U rank sum test applying Statistica software (StatSoft Inc., Tulsa, USA), and differences with $p < 0.05$ were considered significant.

Results

Human NK cells

For a detailed dissection of apoptosis versus lytic function of NK cells, we first established an experimental system to prevent apoptosis in the effector cells. Caspase inhibitors such as ZVAD and DEVD prevent apoptosis induction in other cell systems, and thus, we tested these compounds for their capacity to prevent apoptosis in Jurkat cells and activated NK cells. The addition of 50 or 200 µM ZVAD to Jurkat cells had no influence on proliferation or viability, and likewise, there were no significant differences in viability between NK cells with or without caspase inhibitor treatment (Fig. 1). Following exposure to 1 µM daunorubicin ZVAD completely prevented daunorubicin-induced apoptosis in Jurkat cells in a dose-dependent manner (Figs. 1 and 2). In contrast to Jurkat cells, however, ZVAD alone had no major influence on chemotherapy-induced apoptosis in NK cells (Fig. 1) but combination of 200 µM ZVAD plus 800 µM DEVD largely prevented apoptosis in these cells (Figs. 1 and 2).

Consecutively addressing target cell-induced apoptosis, we found that K562 and ML2 cells provided pronounced induction of NK cell apoptosis, whereas, Jurkat cells had

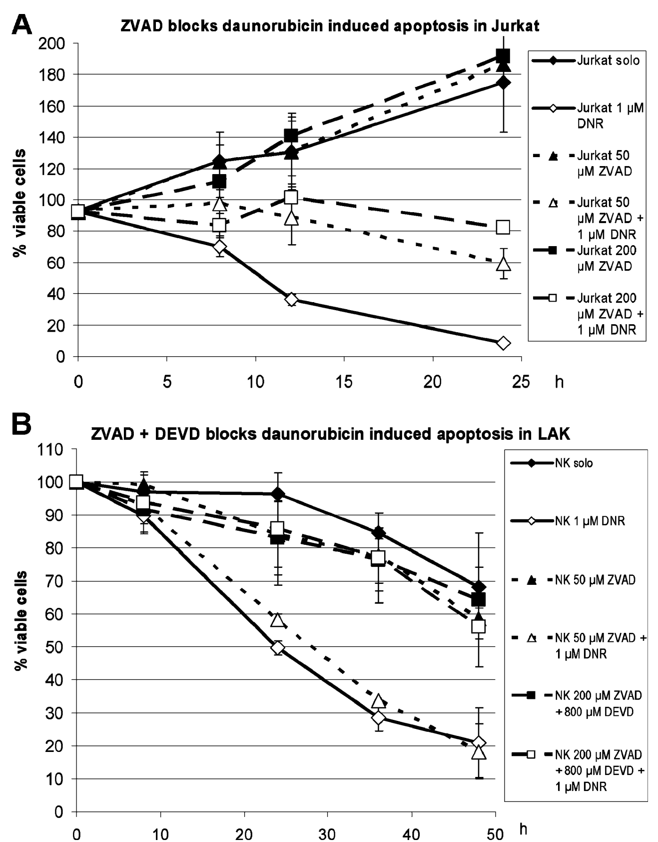

Fig. 1 Caspase inhibitors protect from cytostatic drug induced cell death. Influence of incubation with indicated doses ZVAD (and DEVD) on proliferation and viability of T cell line **a** Jurkat and **b** LAK following incubation with or without 1 µM daunorubicin. LAK were immunomagnetically enriched human NK cells (purity >90%) after short time incubation with IL-2 and -12. Viable cells are defined as annexin V and PI negative. Average cell numbers and standard deviation of viable cells at indicated time points in percent compared to start of experiment (0 h). Jurkat and LAK cells differ in behavior over time. So, different appropriate time points were chosen. Data from four independent experiments are shown

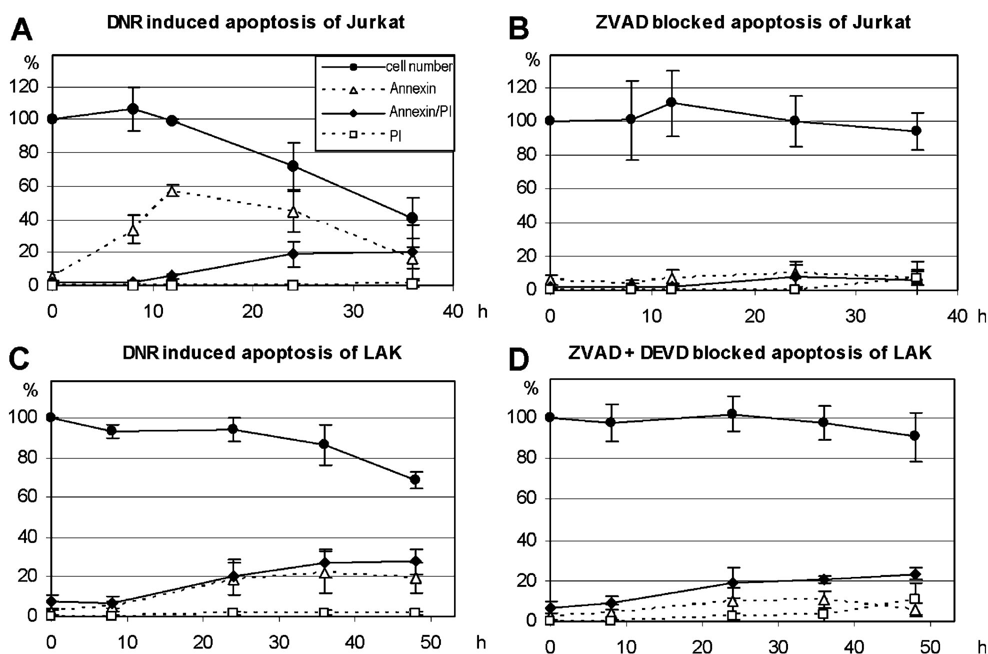

Fig. 2 Caspase inhibitors prevent apoptosis. Influence of incubation with 1 μM daunorubicin on T cell line **a** Jurkat and **c** LAK without and with prior incubation with **b** 200 μM ZVAD and **d** 200 μM ZVAD plus 800 μM DEVD, respectively. LAK were immunomagnetically enriched human NK cells (purity >90%) after short time incubation with IL-2 and -12. Average cell numbers and standard deviation, annexin V positive (apoptotic) and PI positive (necrotic) cells from four independent experiments are shown. Because of slower kinetic of apoptosis, induction in LAK compared to Jurkat, observation periods are altered according to the different cell types

moderate effects. At effector to target ratios of 10:1, 1:1, and 1:10 K562-induced apoptosis in NK cells at proportions of in 14(±6)%, 33(±9)%, and 45(±3)% ML2-induced NK cell apoptosis, in 9(±3)%, 26(±6)%, and 58(±10)% Jurkat-induced NK cell apoptosis, and in 9(±1)%, 10(±5)%, and 24(±5)% of cells, respectively. To shield NK cells from target cell-induced apoptosis, we preincubated the cells with optimal concentrations of ZVAD and DEVD. Addition of ZVAD and DEVD significantly diminished NK cell apoptosis induced by K562 cells (6 ± 3%, 20 ± 10%, and 28 ± 8%) and had only minor or no effect on NK cell apoptosis exerted by ML2 cells (3 ± 3%, 18 ± 3%, and 53 ± 4%) and Jurkat cells (8 ± 5%, 9 ± 5%, and 25 ± 4%), respectively (Fig. 3).

At effector to target ratios of 10:1, 1:1, and 1:10 NK cells induced apoptosis to a greater amount in K562 cells (93 ± 5%, 61 ± 5%, and 11 ± 3%) than in Jurkat cells (75 ± 10%, 42 ± 13%, and 13 ± 9%). Apoptosis induction in ML2 was poor (44 ± 4%, 18 ± 4%, and 6 ± 1%). Importantly, caspase inhibition by ZVAD or DEVD in NK cells had no significant effect on cytolytic activity (Fig. 4). Finally, we analyzed the effect of bystander target cells on the efficacy of NK cell-mediated K562 leukemia cell lysis in cold target inhibition assays. In such assays, bystander cells of both the K562 and ML2 cell line were able to inhibit NK-92-induced lysis of K562 target cells. The inhibition of K562 cell lysis at an effector to target ratio of 1:1 amounted to 41 ± 13% in case of Jurkat bystander cells, 62 ± 22% in case of K562 bystander cells, and 48 ± 22% in case of ML2 bystander cells (Fig. 5).

Thus, leukemic target cells efficiently induce NK cell apoptosis, which can be, in part, overcome by caspase inhibition in the effector cells.

Murine NK cells

Our findings in the human system raised our interest in a murine model system. Thus, we performed cytotoxicity assays of murine NK cells against the lymphoblastic cell lines A20, YAC-1, and WEHI-3. We found efficient lysis of YAC-1 cells, as well as less pronounced of A20 cells, and only poor lysis of WEHI-3 cells. In this setting, NK cell activation by IL-12 and IL-18 with or without IL-7 resulted in significantly higher cytotoxic activity than activation by IL-2 and IL-7 (Fig. 6).

At effector to target ratios of 50:1, 25:1, and 10:1 average lysis of tumor cells in three independent experiments were as follows: A20, 75%, 62%, and 58% (IL-12+18) and 70%, 56%, and 51% (IL-12+18+7) versus 31%, 15%, and 6% (IL-2+7); YAC-1, 81%, 76%, and 71% (IL-12+18) and 72%, 76%, and 55% (IL-12+18+7) versus 42%, 31%, and 20% (IL-2+7); and WEHI-3, 26%, 28%, and 19% (IL-12+18) and 49%, 58%, and 52% (IL-12+18+7) versus 16%, 13%, and 19% (IL-2+7). Notably, there was no evidence for relevant tumor cell-induced apoptosis of cytokine-activated murine NK cells coincubated with A20,

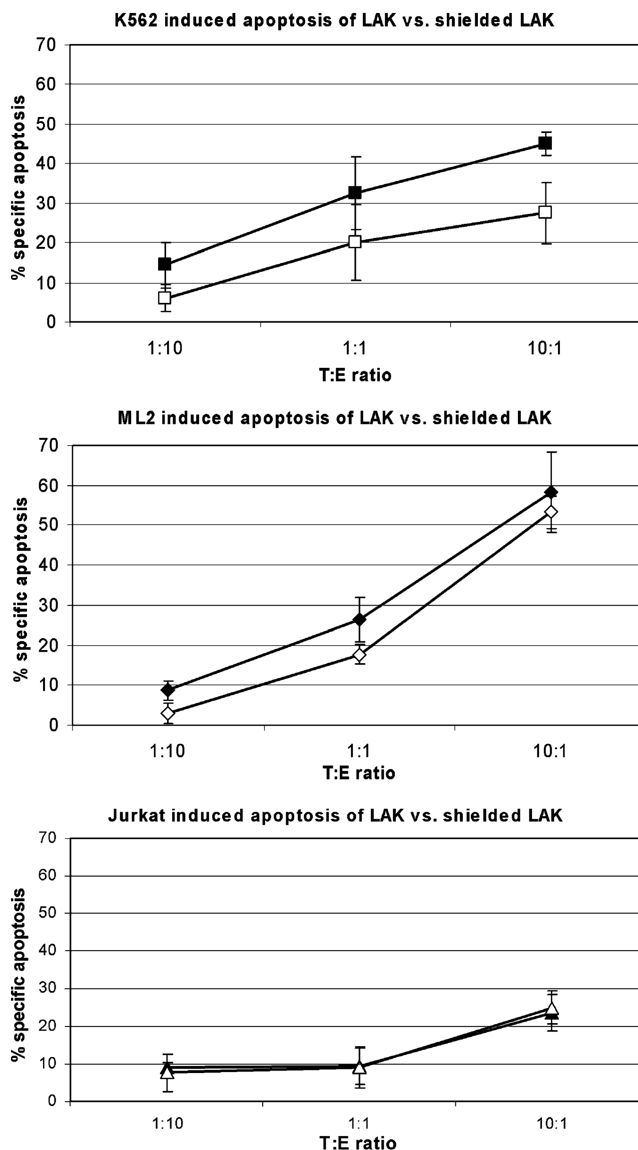

Fig. 3 Impact of caspase inhibitors on target cell-induced apoptosis in LAK cells. Comparison of target cell-induced apoptosis of untreated LAK (*full symbols*) and shielded LAK (*open symbols*) indicates a significant decrease in K562 and a less pronounced or no effect in ML2- and Jurkat-induced apoptosis of shielded LAK. LAK were immunomagnetically enriched human NK cells (purity >90%) after short time incubation with IL-2 and -12

YAC-1, or WEHI-3, respectively (Fig. 6). Thus, in the murine in vitro model, we found no equivalent to target cell-induced apoptosis as described for activated human NK cells in vitro.

Discussion

Target cell-induced apoptosis of NK cells and T lymphocytes has been proposed as a relevant mechanism of tumor cells to escape from the attack by immune cells [13–16].

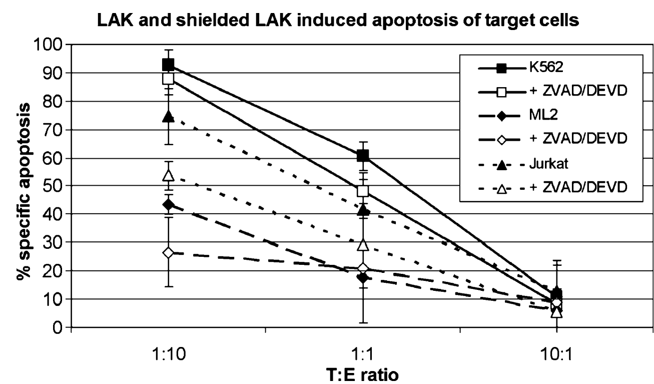

Fig. 4 Effect of shielding against target cell-induced apoptosis on cytotoxic activity. Comparison of efficiency in inducing target cell apoptosis of untreated LAK (*full symbols*) and shielded LAK (*open symbols*) indicates no improvement of cytotoxicity by shielding LAK. Apoptosis induction by LAK (with or without ZVAD/DEVD pretreatment) of K562 is *high*, ML2 *poor*, and Jurkat *intermediate*. LAK were immunomagnetically enriched human NK cells (purity >90%) after short time incubation with IL-2 and -12

Thus, caspase inhibitor treatment of $\gamma\delta$ T cells reduced activation-induced cell death triggered by Fas/Fas-ligand and increased antilymphoma efficacy [24]. Here, we addressed the functional consequences of TiA in NK cells, and we confirmed that malignant target cells caused apoptosis in activated human NK cells [14, 16]. However, we also found that there was no direct association of the susceptibility of the target cells to NK cell-mediated lysis and their potential to induce apoptosis in NK cells. K562 and ML2 cells showed the highest potential to induce apoptosis in NK cells. However, while K562 cells were highly sensitive to NK cell-mediated killing, the cytolytic efficacy of NK cells against ML2 was limited. In previous studies, we had demonstrated that target cell recognition, and NK cell activation elicited by K562 and ML2 cells were similar [8]. However, ML2 cells were found resistant to lysis mainly due to deficient perforin binding [8, 10]. Overall, our results are in line with previous findings that

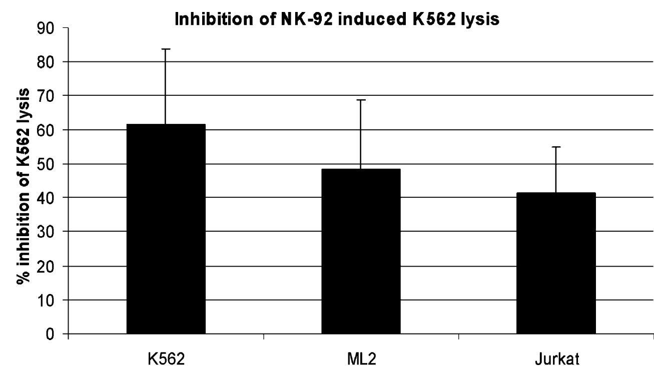

Fig. 5 Results from cold target inhibition assays. Inhibition of NK-92-induced K562 lysis at effector to target ratio of 1:1 due to pre/coincubation with 5×10E4 target cells, as indicated, showed no significant differences (averages and SD of three independent experiments)

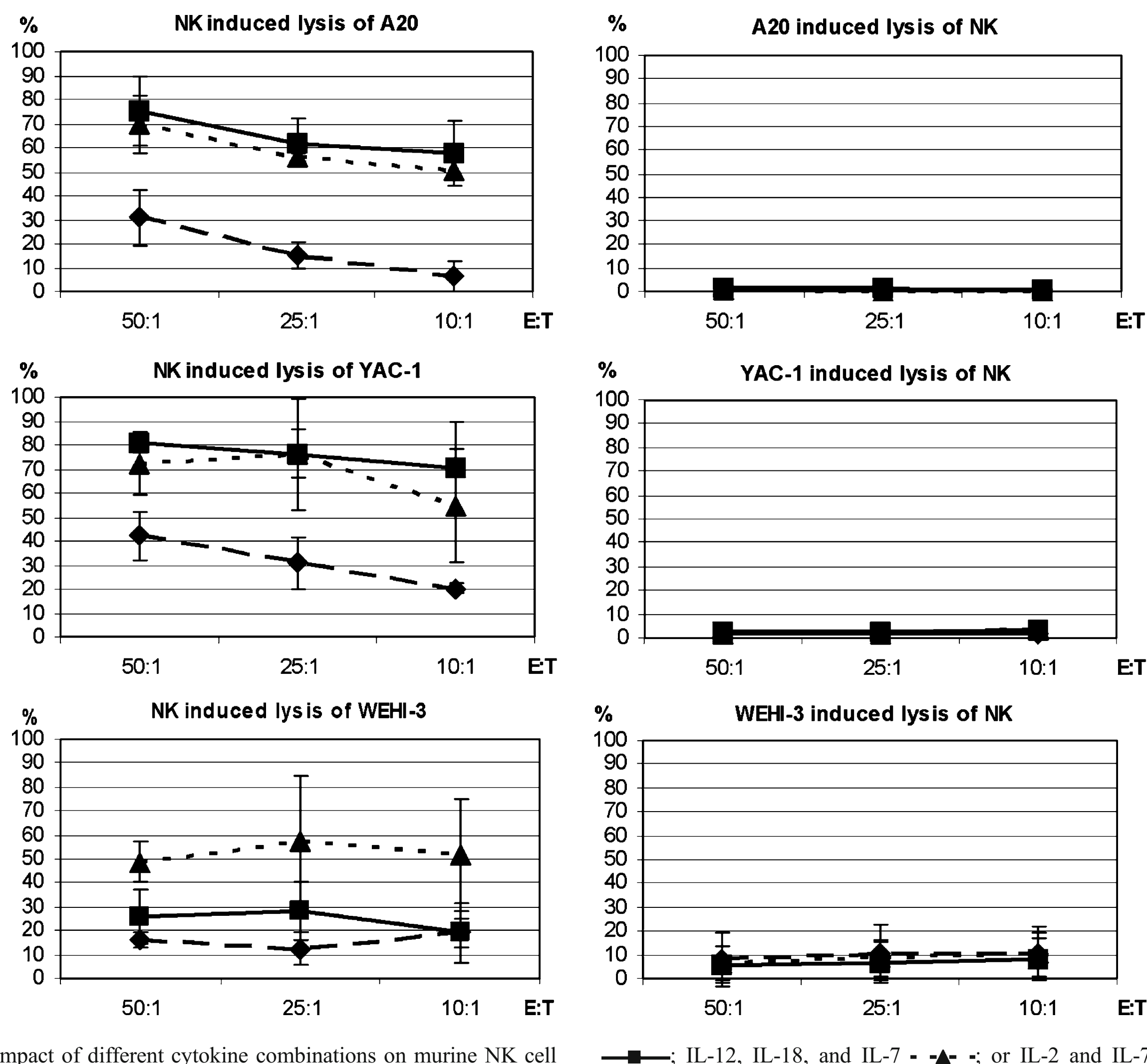

Fig. 6 Impact of different cytokine combinations on murine NK cell cytotoxic efficacy and target cell-induced lysis of murine NK cells. Comparison of NK-induced lysis of A20, YAC-1, and WEHI-3 target cells. Murine NK cells were obtained as described in "Materials and methods" section. NK cells were activated by IL-12 and IL-18 —■—; IL-12, IL-18, and IL-7 - -▲- -; or IL-2 and IL-7 —◆—. Simultaneously, lyses of NK cells were assessed. No significant target cell-induced lysis of murine NK cells is detected. Average lyses and SD from three independent experiments at indicated effector to target ratio (E:T) are shown

TiA is a consequence of NK cells activation and intracellular leakage of granzyme B or Fas-triggered apoptosis [22, 23].

To the best of our knowledge, the data of this study represents the first results on TiA in the murine system. Of the three target cell lines with different susceptibilities to LAK cell lysis, neither the highly susceptible cell line YAC-1, nor the moderately susceptible cell line A20, nor the resistant cell line WEHI-3 showed significant potential for inducing apoptotic death in murine LAK cells. Activating cytokines such as IL-2 and IL-12 have been demonstrated to induce apoptosis in NK cells and T cells and to alter the susceptibility to TiA, whereas, IL-7 was claimed to protect against cytokine-induced apoptosis [30–32]. In our experiments, IL-12 and IL-18 activated murine NK cells more efficiently than IL-2. IL-7 further enhanced NK cells activity against the relatively resistant cell line WEHI-3. However, none of these cytokines rendered NK cells susceptible to TiA. Thus, our data do not support the notion of TiA contributing to target cell resistance in the murine system.

Caspase-3 mediated signaling has been shown to play a pivotal role in TiA [23]. In addition, $\gamma\delta$ T cells could be successfully protected against activation-induced apoptosis, resulting in enhanced cytotoxicity against malignant lymphoma cells [24]. Thus, shielding of NK cells against apoptosis by preincubation with caspase inhibitors might be envisioned to inhibit TiA and to augment cytolytic activity. Following this approach, ZVAD, a broad caspase inhibitor with focus on caspase-1 [33], partially protected NK cells from daunorubicin-induced apoptosis. However, with addition of the caspase-3, -1, and -7 inhibitor DEVD [34, 35], complete protection of NK cells from daunorubicin-induced apoptosis was achieved. Surprisingly, caspase inhibition did

not prevent TiA completely. Intracellular signaling pathways of apoptosis depend on the trigger mechanism and differ between cell types. TiA in NK cells seems to be partly independent of caspase pathways. Complete inhibition of TiA must probably include blocking of alternative apoptosis pathways. Others have reported that blocking of tyrosine kinases with inhibitors, like herbimycin A, may prevent TiA [16, 20]. However, herbimycin A may influence the cytotoxic potential of NK cells by inhibiting NK cells activation.

Preincubation with ZVAD and DEVD did not result to an improved NK cell-mediated cytolysis of both susceptible and resistant target cell lines. This finding, together with our results in the murine system, is a strong argument against TiA as a major factor of resistance against NK cells. One reason why shielding of NK cells against TiA may not be followed by enhanced cytotoxicity could be that any NK cell, once activated by an appropriate target, is not able to exert a second cytotoxic hit against a second target. Alternatively, protection from TiA with caspase inhibitors could interfere with NK cells cytotoxic efficacy. ZVAD and DEVD treatments may reduce NK cells cytotoxic reactivity. If so, protected NK cells could not exert enhanced cytotoxicity despite prevention of TiA. But, in our cold target inhibition experiments, preceding incubation with various target cell lines significantly diminished NK cell activity even against highly susceptible targets such as K562 cells. Thus, consumption of NK cells cytotoxic potential during first target cell contact makes the number of surviving NK cells, after first contact, an irrelevant determinant of NK cells cytolytic efficacy. We used NK-92 cells as effector cell population in the cold target inhibition experiments. NK-92 cells are not regulated by components of the killer cell immunoglobulin-like receptor (KIR) system, as they are devoid of KIR expression except KIR2DL4. Thus, differences in susceptibility to NK-92 cell-mediated lysis depend on activating receptors, e.g., NCR and NKG2D (afferent resistance), or on resistance to the cytolytic mechanisms of NK cells, e.g., perforin, granzymes, and Fas-ligand (efferent resistance). The cell lines used in our experiments differed in susceptibility to NK cell-mediated lysis, but they uniformly exhausted the cytotoxic potential of the NK cells. This finding suggests a major role of efferent resistance in our in vitro models rather than afferent deficits.

In conclusion, we found that target cells susceptibility to NK cell-mediated cytotoxicity was not associated with TiA and that TiA was absent in our murine model system. Furthermore, we found that pharmacological inhibition of TiA did not improve lytic activity of NK cells and that NK cells loose cytotoxic capacity during first encounter of target cells. Taken together, our results raise doubts of a relevant role of TiA as a mechanism of resistance. Other mechanisms must contribute to resistance against NK cells cytotoxicity. Namely, these are afferent and efferent deficit by either failure of target cell recognition or failure of NK cells cytotoxic arsenal [6–12]. To overcome target cell resistance against NK cells, future efforts should focus on the mechanisms of efferent resistance and on the role of inhibitory and stimulatory killer cell receptors.

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