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Human NK cells activated by EBV^{+} lymphoblastoid cells overcome anti-apoptotic mechanisms of drug resistance in haematological cancer cells

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Keywords: apoptosis, B lymphoblastoid cell line, haematological neoplasia, multidrug acquired resistance, NK cells

Abbreviations: B-CLL, B cell chronic lymphocytic leukemia; EBV, Epstein-Barr virus; IAP, inhibitor of apoptosis; KIR, killer inhibitory receptor; LCL, lymphoblastoid B cell line; NK, natural killer; NKR, NK cell receptor; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; Tc, cytotoxic T.

Natural killer (NK) cells recognize and eliminate transformed or infected cells that have downregulated MHC class-I and express specific activating ligands. Recent evidence indicates that allogeneic NK cells are useful to eliminate haematological cancer cells independently of MHC-I expression. However, it is unclear if transformed cells expressing mutations that confer anti-apoptotic properties and chemoresistance will be susceptible to NK cells. Allogeneic primary human NK cells were activated using different protocols and prospectively tested for their ability to eliminate diverse mutant haematological and apoptotic-resistant cancer cell lines as well as patient-derived B-cell chronic lymphocytic leukemia cells with chemotherapy multiresistance. Here, we show that human NK cells from healthy donors activated *in vitro* with Epstein Barr virus positive (EBV^{+})-lymphoblastoid cells display an enhanced cytotoxic and proliferative potential in comparison to other protocols of activation such a K562 cells plus interleukin (IL)2. This enhancement enables them to kill more efficiently a variety of haematological cancer cell lines, including a panel of transfectants that mimic natural mutations leading to oncogenic transformation and chemoresistance (e.g., overexpression of Bcl-2, Bcl-X_L and Mcl-1 or downregulation of p53, Bak/Bax or caspase activity). The effect was also observed against blasts from B-cell chronic lymphocytic leukemia patients showing multi-resistance to chemotherapy. Our findings demonstrate that particular *in vitro* activated NK cells may overcome anti-apoptotic mechanisms and oncogenic alterations frequently occurring in transformed cells, pointing toward the use of EBV^{+}-lymphoblastoid cells as a desirable strategy to activate NK cells *in vitro* for the purpose of treating haematological neoplasia with poor prognosis.

Introduction

Mutations in proteins or pathways that regulate apoptosis are common in different types of cancer, including p53, caspase inhibitors (inhibitors of apoptosis [IAPs]) and Bcl-2 family members. The last is a family of proteins that controls the mitochondrial intrinsic pathway of apoptosis consisting of anti-apoptotic (i.e., Bcl-2, Bcl-X_L, Mcl-1 or A1) and pro-apoptotic (i.e., Bak, Bax, Bim, Bid, PUMA or Noxa) proteins, which antagonize each other through heterodimerization.[1] Mutations affecting the Bcl-2 family (including its transcriptional regulation by members of the p53 family) are of special relevance in tumors from haematological origin. Several anti-apoptotic proteins including Bcl-2, myeloid-cell leukemia 1 (Mcl-1) and BCL2-like 1 (BCL2L1, better known as Bcl-X_L) have been found to be overexpressed and to contribute to tumor initiation, progression, and therapeutic resistance in several haematological malignancies, such as lymphoma, chronic lymphocytic leukemia (CLL) or acute myeloid leukemia (AML).[2-8] Recently it has been shown that somatic mutations in human cancer patients associated to MCL1 and BCL-X_L

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overexpression are a common feature in several types of cancer of haematological origin.[9]

During the last years, novel therapies have focused on the manipulation of the immune response to eliminate chemotherapy-resistant tumor cells, as well as metastatic cells. Most of them are directed to break immune tolerance and, ultimately, enhance the recognition and elimination of tumor cell by $CD8^+$ cytotoxic T (Tc) cells.[10,11] Indeed, we have previously shown in mouse models that activated Tc cells are capable of killing transformed cells with mutations impacting the apoptotic machinery.[12] These findings indicate that cytotoxic lymphocytes could constitute an efficacious therapeutic approach to treat tumors that fail to respond to chemotherapy or radiotherapy. However, Tc cell killing depends on the presence of tumor-associated antigens presented by MHC class-I (MHC-I) to recognize their target, a presentation that cancer cells avoid by a variety of means.[11]

Natural Killer (NK) cells were originally identified as the lymphocyte lineage responsible for the natural toxicity observed against virally infected, transformed, or allogeneic target cells.[13,14] Subsequently, NK cells were characterized as the immune cell type responsible for killing cells that have downregulated MHC-I expression.[15] NK cells constitute a phenotypically heterogeneous population with distinct functional characteristics that include but are not limited to the execution of cell killing.[16] Activation of NK cells is regulated both by the level of MHC-I expression on target cells as well as the expression of NK cell receptor (NKR)-activating ligands that are usually upregulated during infection or transformation. In this way, the balance between inhibitory and activating signals transduced respectively by inhibitory and activating NKRs will dictate the fate of NK-cell activation and, subsequently, target defective cells for elimination.[17,18]

Among the most prominent inhibitory NKRs are the KIR (killer inhibitory receptor) family, whose members show extensive polymorphism and recognize shared epitopes present in MHC-I molecules. During development NK cells are educated in the context of self MHC-I molecules. Therefore, in allogeneic settings and in a context of KIR-MHC mismatch, KIR ligands may not inhibit NK cell activation.[19] Indeed, allogeneic NK cells are able to eliminate haematologic cancer cells irrespectively of MHC-I expression[19,20] and generate improved prognosis in the context of mismatched haematopoietic transplantation.[21,22] Hence, they could be a good alternative for the treatment of various haematological neoplasms.[23-27]

The previously mentioned studies have used unstimulated NK cells. However NK-cell activation increases NK-cell cytotoxicity, which may be a crucial step to treat cancer cells with enhanced resistance to cell death. Several protocols to expand and/or activate NK cells [28] have been developed, including unique combinations of exogenous cytokines in the presence or absence of stimulating cells such as the MHC-I deficient cell K562, peripheral blood mononuclear cells, or transformed B cell lines. Among them Epstein-Barr virus (EBV) positive lymphoblastoid B cell lines (LCLs) have been the common NK-cell proliferation and activation standard since this protocol was introduced in the late 1980s.[29] Although the different protocols of activation produce NK cells with enhanced cytotoxic potential, we have recently shown that changes in the activation protocol produces changes in the NK cell transcriptome that could affect the quality of the NK cells generated, crucially including cytotoxic potential against target cells.[30]

Here, we investigated the potential of healthy donor-derived NK cells activated via an EBV^+-LCL alone (i.e., in the absence of exogenous cytokines) to selectively kill haematological cancer cells and mutant leukemia cell lines, particularly those with a multidrug resistant phenotype acquired by mutations in various Bcl-2 family members.

Results

R69-LCL activates human NK cells more efficiently than stimulation with K562 and exogenous IL2

We have recently published that encounter of NK cells with distinct targets, such as interleukin (IL)2 stimulated-K562 leukemia (K562/IL2) *vs* EBV^+-LCL (R69-LCL), induces specific genomic remodelling.[30] However, this previous work did not investigate whether these unique encounters induce different patterns of cytolytic behavior. To address this, we first compared the ability of enriched NK cells from healthy donors differentially activated under the 2 different stimuli (K562/IL2 and R69-LCL) to kill haematological cancer cell lines of diverse origins. We chose K562 cells since recent studies used this cell line together with cytokines such as IL2, IL15 or IL21 as an alternative to EBV^+-LCLs to support NK-cell activation.[28,31-33] To generate activated NK cells, peripheral blood mononuclear cells (PBMCs) were cultured under the different stimuli and 5 days later NK cells were magnetically isolated by MACS cell separation, as indicated in Materials and Methods.

As shown in **Figure 1A**, these activated NK cells were capable of killing the chronic myeloid leukemia line K562, as well as the Burkitt's lymphoma Raji and the acute myeloid leukemia U937. Although NK cells activated under both protocols efficiently killed target cells, cytotoxicity was significantly higher when R69-LCLs were used as the NK cell-activating stimulus. Activation of NK cells with K562 cells together with both IL2 and IL15 did not increase the cytotoxicity of NK cells activated with K562 and IL2 alone (**Fig. S1**), confirming previous data indicating that IL15 does not enhance the stimulatory potential of IL2 on the activation of NK cell cytotoxicity.[34] NK cells activated with a HLA-I deficient EBV^+-LCL, 721.221 ([35,36] and **Fig. S2**), induced target cell death to the same extent as NK cells activated via exposure to R69-LCL (**Fig. 1B**), suggesting that the effect observed was not specific for R69-LCL and not influenced by HLA-I levels. Note that a small degree of binding of the HLA-ABC antibody W6/32 to HLA-I deficient 721.221 cells has been previously reported [37] that is independent of HLA-ABC expression.[36]

In addition, we tested the proliferative abilities of NK cells in the diverse PBMCs cultures activated by exposure to the different stimuli (i.e., R69, K562, R69/IL2 and K562/IL2) and found that NK cells did not proliferate in the PBMC cultures stimulated

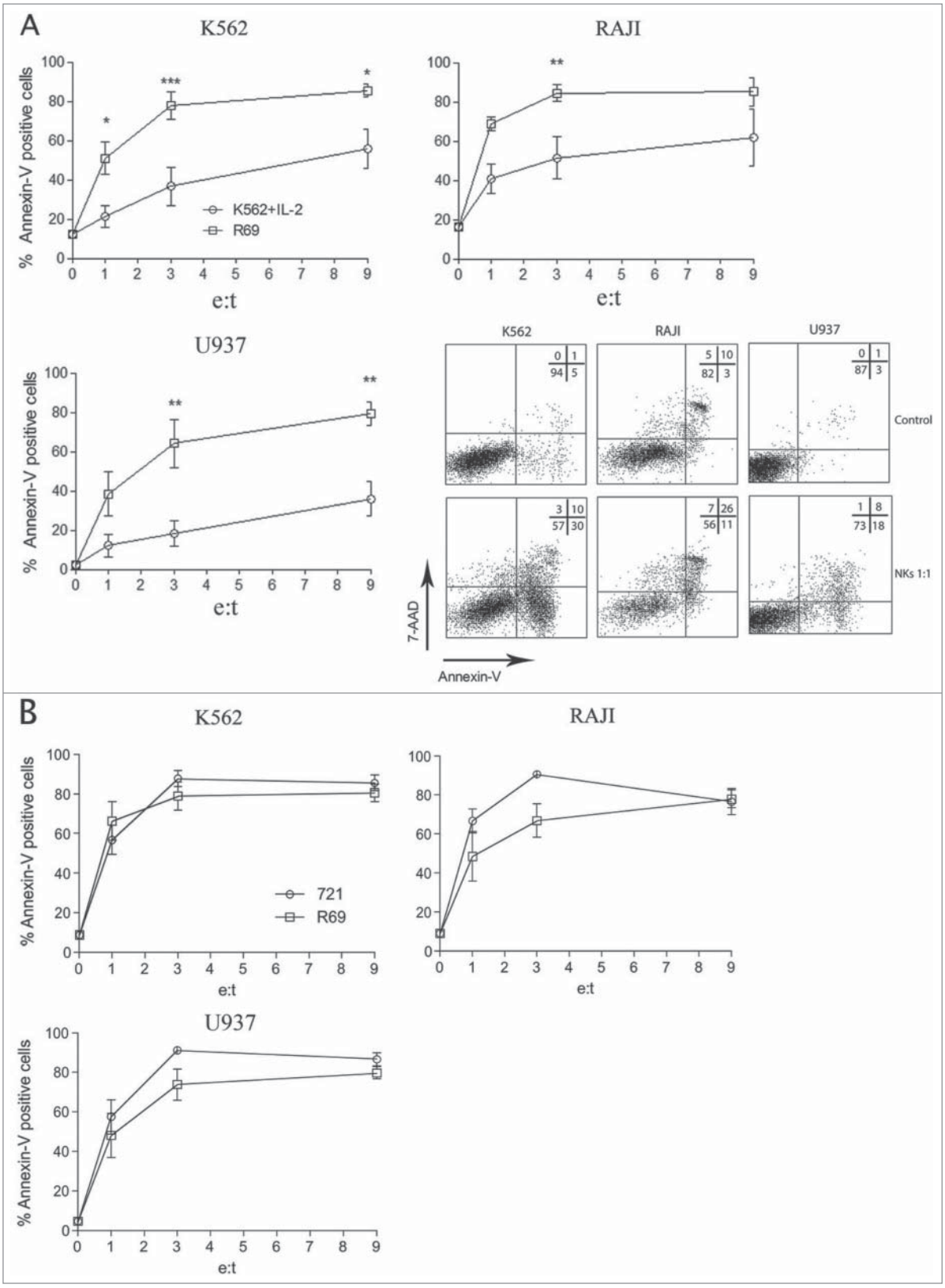

Figure 1. For figure legend, see page 4.

with K562 in the presence or absence of IL2 after 2 weeks. In contrast, the absolute number of NK cells in the PBMC cultures stimulated with R69 or R69 plus IL2 increased over 50 times. This finding indicates that, in addition to the cytotoxic potential, stimulation with R69 cells enhances the proliferative potential of NK cells in comparison with K562 cells.

Based on these results, NK cells activated with R69-LCL or K562 and IL2 were used in the next experiments.

In **Figure 1** and in the following experiments cell cytotoxicity was evaluated by analyzing phosphatidyl-serine translocation (Annexin V staining) and cell membrane permeabilization (7-aminoactinomycin D [7AAD] staining) as indicated in Materials and Methods (see dot plots in **Fig. 1A**). Results in the graphs were represented as the % of cells positive for Annexin V staining including those that have incorporated 7AAD (Annexin V^+/$7AAD^{-}$ $^{+}$ Annexin $V^+/7AAD^+$). Cells staining only with 7AAD were not significantly detected in any case.

Soluble ICAM-1 (CD54) inhibited phosphatidyl-serine translocation ([38] and data not shown) confirming that cell death was dependent on cell-to-cell contact mediated by ICAM-1 ligation with integrin (LFA-1). Since NK cell mediated-cytotoxicity was significantly reduced if NK cells were activated in the absence of other PBMC population (**Fig. S3**), we used NK cells activated in the presence of PBMCs in the subsequent experiments.

R69-LCL stimulated NK cells up regulate granzyme B expression

Next we tested the phenotype of NK cells activated during co-culture of PBMCs and R69-LCL (**Fig. 2A**). After 5 days of activation with either K562/IL2 or R69-LCL, the intensity of CD56 staining among stimulated NK cells was clearly enhanced. In addition to the population of cells expressing both CD56 and CD16, we observed a new population expressing CD56 but not CD16 that could represent cells that had lost CD16 by virtue of shedding, as previously described.[39]

When the expression of cytotoxic molecules in $CD56^+CD3^-$ cells was analyzed (**Fig. 2B**) it was found that resting NK cells mainly expressed perforin (PRF1, or Perf), granzyme (Gzm)A and GzmB, with low expression of GzmK or granulysin (GNLY) (**Fig. 2B**). Activation of NK cells did not induce an increase in the percentage of cells expressing these molecules (**Fig. 2B**), but, remarkably, enhanced the intracellular levels of GzmB (**Fig. 2C**), correlating with the observed increase in cytotoxic potential.

Finally, we tested the profile of NKR expression in un-stimulated and activated NK cells. As shown in **Figure 2D**, varying percentages of un-stimulated NK cells expressed the NK cell inhibitory receptors NKG2A and ILT2, as well as the activating receptors NKG2D, DNAM1, NKp30 and NKp46. The expression of KIRs was variable with a predominant expression of KIR2DL2/L3/S2. As expected NKp44 was only expressed in activated NK cells.[40] There were slight differences in the expression of specific NKRs between K562/IL2 activated NK cells and those stimulated by R69-LCL. During activation with R69-LCL, we found that: i) the expression of inhibitory receptor NKG2A as well as the activating receptors DNAM1 and NKp30 were upregulated; ii) the expression of the activating receptor NKp46 did not change and iii) the expression of the inhibitory receptor ILT2 was downregulated.

NK cells activated with R69-LCL preferentially use granule exocytosis to kill target cells

Next we analyzed whether these 2 groups of activated NK cells use granule exocytosis and/or death ligands to kill target cells. We used Jurkat cells as targets since they are sensitive to both granule exocytosis and death receptor pathways[41] and since we could also employ available Jurkat sublines to analyze the effect of anti-apoptotic mutations on NK-mediated cell death. Against Jurkat cells, we first confirmed that the cytotoxic potential of NK cells activated with R69-LCL was indeed higher than that of NK cells activated with K562/IL2 (**Fig. 3A**). **Figure 3B** shows that Jurkat cells overexpressing the death receptor inhibitory cowpox serpin CrmA were as sensitive as parental cells to activated NK cells, arguing against death receptor mediated-apoptosis. As a control Jurkat-CrmA cells were insensitive to Fas- and TRAIL-mediated cell death (data not shown). In addition calcium chelation by EGTA, which is known to inhibit granule exocytosis, completely blocked cell death induced by activated NK cells (**Fig. 3C**). Altogether, these results indicate that activated human NK cells exclusively kill Jurkat target cells by granule exocytosis under the conditions tested.

Activated NK cells efficiently kill mutant cell lines resistant to apoptosis

Next, we sought to test whether naive and/or activated NK cells would be able to overcome drug resistance in haematological cancer cells. To accomplish this, we decided to use mutant cell lines highly resistant to apoptosis induced by a variety of stimuli, including chemotherapy and radiotherapy. First, we employed a panel of mutants of the leukemia cell line Jurkat in which anti-apoptotic or pro-apoptotic proteins of the Bcl-2 family were overexpressed or downregulated, respectively. All the mutant cells have been previously characterized in our group [41] and found to

Figure 1. (See previous page) Human NK cells activated with R69-LCLs present higher cytotoxicity against haematological cancer cellsthan those activated with K562 cells plus IL2. Natural killer (NK) cells were enriched by magnetic isolation (MACS) from peripheral blood mononuclear cells (PBMCs) and activated over the course of 5 days with K562 cells plus interleukin 2 (IL2) (A) or Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs) (A, B) or HLA-I deficient EBV^+-LCL 721.221 LCLs (B). Subsequently, activated NK cells were incubated with K562, Raji and U937 cells at different effector/target (E:T) cell ratios for 4 hours. Phosphatidyl serine plasma membrane externalization (Annexin-V) and membrane permeabilization (7-aminoactinomycin D [7AAD]) were analyzed by 3-color fluorescence cytometry as described in Materials and Methods. A representative experiment is shown in dot plots using NK cells stimulated with K562/IL2 at 1:1 E:T cell ratio. Numbers correspond to the percentage of cells in each quadrant. Results are presented as the mean ±SEM of 5 independent donors from 2 independent experiments. Statistical analysis was performed by 2-way ANOVA with Bonferroni´s post hoc test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

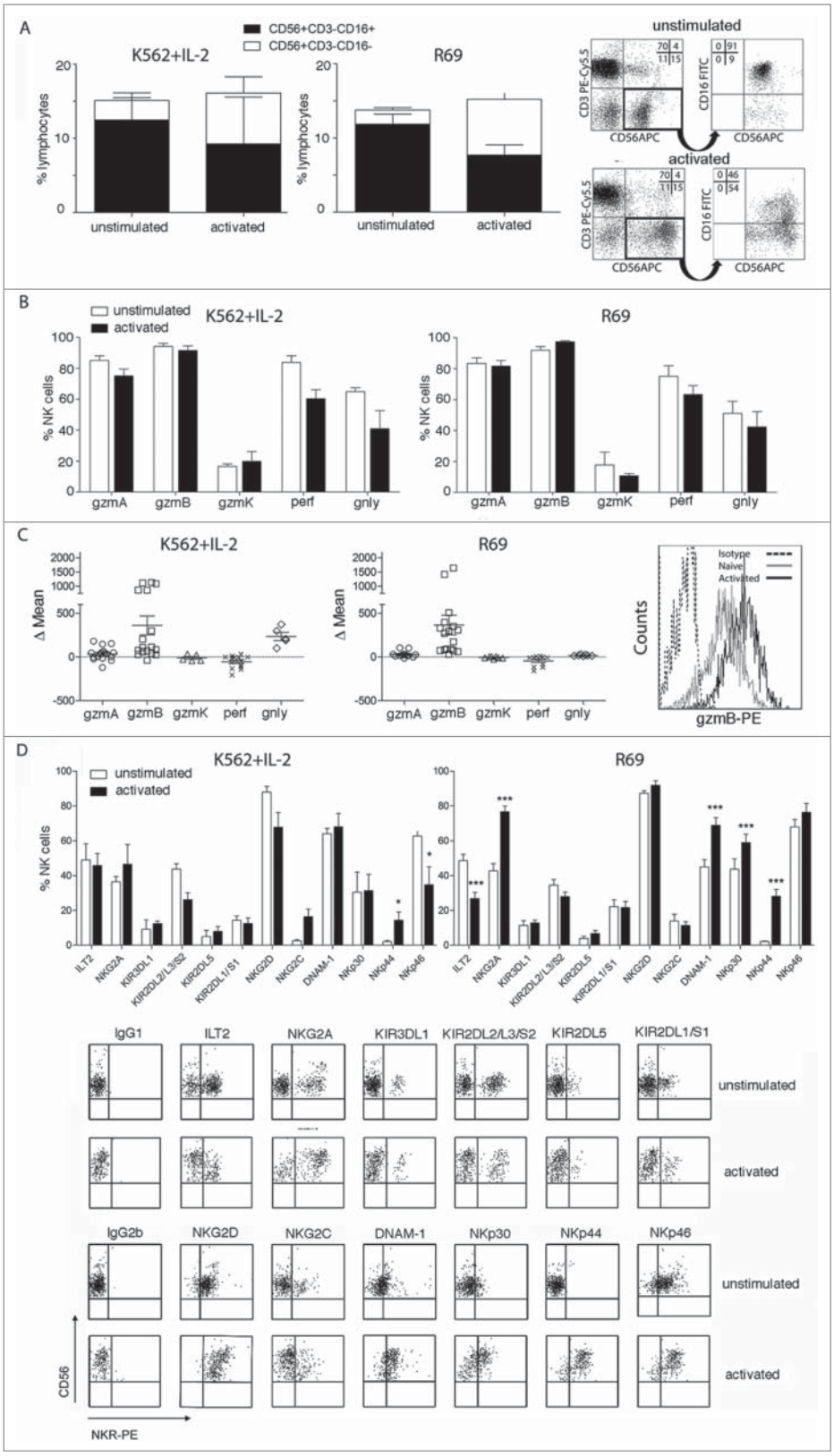

Figure 2. Characterization of NK cells activated with R69-LCLs. Peripheral blood mononuclear cells (PBMCs) were cultured for 5 days in the presence of K562 and interleukin 2 (IL2) or Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs) as described in Materials and Methods. Subsequently, the following parameters were analyzed in activated and unstimulated PBMCs from the same donor by immunofluorescence staining and cytofluorimetric analysis of the indicated profiles. (**A**) Percentage of $CD56^+CD3^-CD16^+$ or $CD56^+CD3^-CD16^-$ cells. Data in the graphics are represented as the mean ± SEM of at least 5 independent donors from at least 3 independent experiments. Dot plots of a representative donor showing the gating strategy are shown on the right panels. Numbers correspond to the percentage of cells in each quadrant. (**B**) Percentage of $CD56^+CD3^-$ NK cells expressing granzyme A (Gzm)A, GzmB, GzmK, perforin (PPerf) and granulysin (Gnly). Results are presented as mean ± SEM of at least 5 independent donors from at least 3 independent experiments. (**C**) The variation in the mean fluorescence intensity (MFI) between activated and unstimulated $CD56^+CD3^-$ NK cells. Each symbol corresponds to an independent donor from 3 independent experiments. The histogram shows the increase in GzmB expression in a representative donor (right panel). (**D**) Percentage of $CD56^+CD3^-$ NK cells expressing the indicated NK receptors (NKRs). Results are presented as the mean ±SEM of at least 5 independent donors from at least 2 independent experiments. Dot plots of a representative donor are shown in the lower panels. Statistical analysis was performed by 2-way ANOVA with Bonferroni´s post hoc test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ indicate statistically significance comparing unstimulated with activated NK cells.

show resistance to several cell death stimuli, including the chemotherapy drugs doxorubicin, vincristine, bortezomib and cladribine (data not shown) and the immune cytotoxic protein TRAIL.[41] In addition to these targeted mutations, Jurkat cells natively express mutant p53 protein and lack Bax expression. Thus, this model can be considered an extreme case of multidrug resistance to apoptosis. Jurkat cells transfected with the corresponding empty vectors (Neo and PLHTVM) were used as control.

As shown in **Figure 4A**, freshly isolated NK cells induce low levels of cell death of parental Jurkat cells, a death induction that was significantly reduced in Jurkat cells overexpressing either Bcl-2 or Bcl-X_L. The same result was obtained when Jurkat cells with down regulated Bak expression were used (**Fig. 4B**). In contrast, overexpression of Mcl-1 or

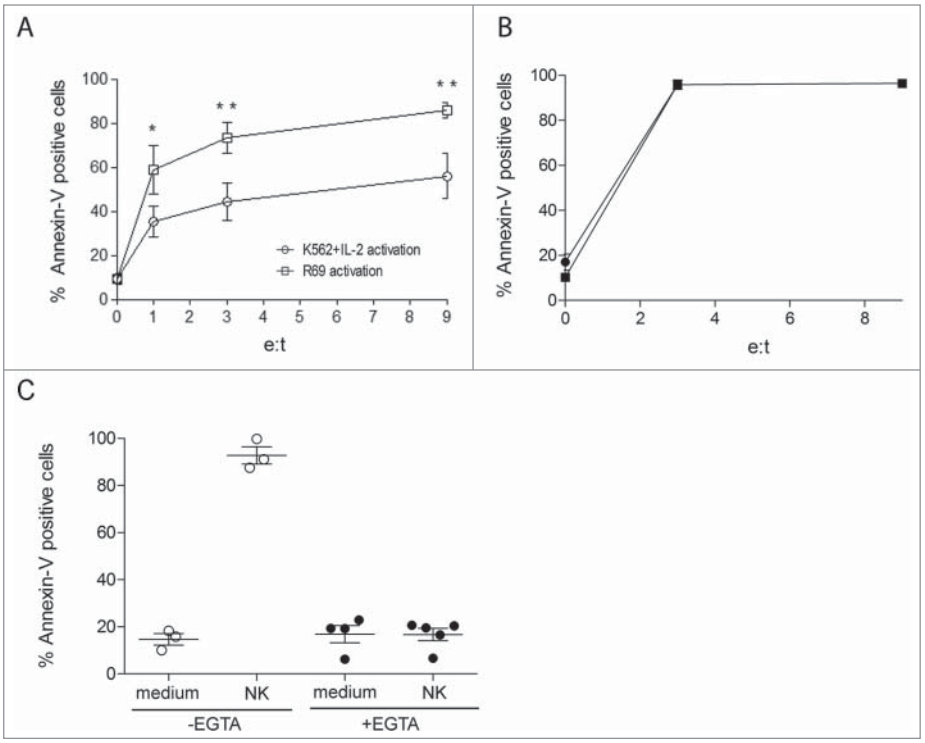

Figure 3. NK cells activated with R69-LCL kill Jurkat target cells via granule exocytosis. (**A–C**) Natural killer (NK) cells were enriched by magnetic isolation (MACS) from peripheral blood mononuclear cells (PBMCs) activated during 5 days stimulation with K562 and interleukin 2 (IL2) (**A**) or Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs) (**A–C**). (**A–B**) NK cells were incubated with Jurkat (circles) or Jurkat.CrmA (squares) cells at the indicated Effector:Target (E:T) cell ratios for 4 hours (**A–B**) or with Jurkat cells in the presence or absence of 1 mM ethylene glycol tetraacetic acid (EGTA; **C**). Phosphatidyl-serine (PS) exposure on the plasma membrane was detected via Annexin-V staining and analyzed by 3-color fluorescence cytometry. Results are presented as mean ±SEM of NK cells derived from 4 independent donors from 2 independent experiments.

downregulation of Bim expression failed to protect Jurkat cells from NK cell-mediated cytotoxicity.

Activation of NK cells *in vitro* greatly increased their cytotoxic potential against Jurkat cells (**Fig. 4A and B**, right panels). Activated NK cells killed all mutant cell lines at the same level as parental cells indicating that mutations in the apoptotic machinery, which increase the resistance to chemotherapy drugs and to naive NK cells, did not affect cell death induced by NK cells activated with R69-LCL. As shown in **Figure 5**, this effect was preserved even with an E:T cell ratio as low as 0.25:1 (i.e., 1 effector cell to 4 target cells) were used as shown in **Figure 5**.

The cytotoxic potential of NK cells activated with R69-LCL is higher than that of those activated by K562 and IL2 (**Figs. 1 and 3**). Thus, we wondered if the stimulus selected to activate NK cells would influence the ability of NK cells to kill cancer cells with resistance to apoptosis. As shown in **Figure 4C and D**, in contrast to R69-LCL, Jurkat cells deficient in Bak or Bim showed a significant resistance to cell death induced by K562/IL2 activated NK cells. These results indicate that NK cells activated by R69-LCL stimulation kill apoptosis-resistant mutants more efficiently than NK cells activated by K562 and IL2 and suggest that the stimulus used to activate NK cells is important to predict the sensitivity of cancer cells with acquired drug resistance to NK cell-mediated cytotoxicity.

Caspase 3 is activated in Jurkat mutant cell lines

We and others have previously shown that cytotoxic cells use Perf and GzmB to activate caspase-3 and induce cell death through 2 major pathways: i) direct activation of caspase-3 after proteolytic cleavage by GzmB; ii) activation of the intrinsic mitochondrial pathway mediated by cleavage of the death agonist Bid.[12,42]

Accordingly, we decided to analyze whether activated NK cells could induce caspase-3 activation in cells overexpressing Bcl-X_L. As shown in **Figure 5A**, activated NK cells were equally capable of inducing caspase 3 activation in Jurkat parental cells and in Jurkat cells overexpressing Bcl-X_L (**Fig. 5A**), indicating that caspase-3 was activated independently of the mitochondrial apoptotic pathway. Even when the E:T cell ratio was reduced to 0.5–0.25:1, the relatively few NK cells present were still able to activate caspase-3 and induce cell death in the Bcl-X_L overexpressing cell line (**Fig. 5B**). The level of caspase 3 activation perfectly correlated with the level of cell death analyzed by phosphatidyl-serine translocation (**Fig. 5B**) suggesting that NK cells were killing Jurkat cells by apoptosis.

Activated NK cells overcome caspase-3 inhibition in parental and mutant cell lines

Our findings indicate that human NK cells activated with R69-LCL overcome chemoresistance by bypassing the mitochondrial apoptotic pathway through the activation of the death effector caspase-3. Importantly, overexpression of the proto-oncogene Bcl-X_L, which completely prevented cell death induced by several chemotherapy drugs (data not shown), did not affect activated NK cell-mediated killing even when cell death of parental cells was lower than 30% (**Fig. 5B**).

Human cancer cells of diverse origin overexpress IAPs that inhibit caspase activity contributing to both oncogenesis and resistance to apoptosis.[43] In light of these findings, we wondered about the potential of human NK cells activated via R69-LCL to kill cells in which all known anti-apoptotic mechanisms were inactivated. To this aim, we analyzed NK-induced cell death of the mutant cell lines in which effector caspases would be broadly inhibited by the cell permeable and irreversible synthetic caspase-3/7 inhibitor Ac-DEVD-

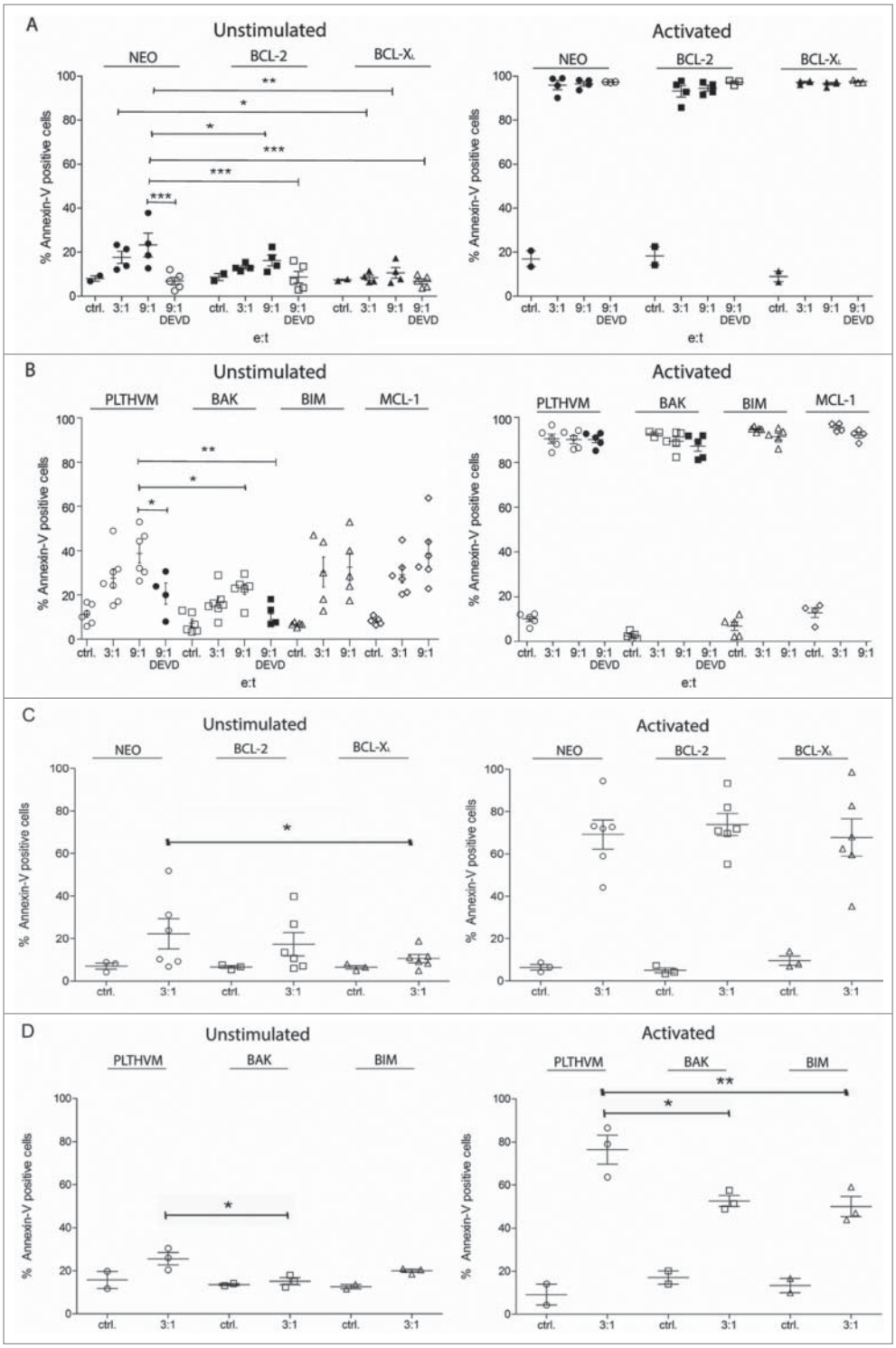

Figure 4. Activated NK cells are capable of killing Jurkat cell mutants with resistance to apoptosis. **(A, B)** Natural killer **(**NK) cells were enriched by magnetic isolatin (MACS) from freshly isolated peripheral blood mononuclear cells (PBMCs) or from PBMCs activated during 5 days stimulation with Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs). NK cells were then incubated with Jurkat Neo/PLTHVM cells (control) or with the indicated mutants for 4 hours at 9:1 and 3:1 (**A**) and (**B**) effector/target cell ratios. Phosphatidyl-serine (PS) exposure on the plasma membrane was detected via Aannexin-V immunostaining and analyzed by 3-color fluorescence cytometry. (**A, B**) Results are presented as the mean ±SEM of NK cells derived from at least 4 independent donors performed in 2 independent experiments in (**A**) and 7 (Bak and Bim) or 4 (Mcl-1) independent donors in 3 independent experiments in (**B**). (**C, D**) NK cells were enriched by magnetic isolation (MACS) from freshly isolated PBMCs or from PBMCs activated during 5 days with K562 plus interleukin 2 (IL2). NK cells were then incubated with Jurkat Neo/PLTHVM cells (control) or with the indicated mutants for 4 hours at 3:1 effector/target ratio. PS exposure on the plasma membrane (Annexin-V) was analyzed by 3-color fluorescence cytometry. (**C, D**) Results are presented as mean ±SEM of 6 independent donors performed in 3 independent experiments in (**C**) and 3 independent donors in 3 independent experiments in (**D**). Each symbol corresponds to an independent NK cell donor. Statistical analysis was performed by 1-way ANOVA with Bonferroni´s post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

fmk. The presence of Ac-DEVD-fmk was sufficient to nearly abrogate unstimulated NK cell-induced target cell death of parental Jurkat cells or of the various mutant cells lines (**Fig. 5A and B**); Jurkat cell death induced by an anti-Fas cytotoxic antibody was also sensitive to Ac-DEVD-fmk (data not shown). In contrast, this inhibitor had no effect on cell death induced by NK cells activated with R69-LCL (**Fig. 5A and B**, right panels).

Activated NK cells kill other types of haematological cancer cell lines overexpressing proto-oncogenes of the Bcl-2 family

Finally, in order to extend the relevance of our results to other types of haematological cancer cells, we analyzed if NK cells stimulated with R69-LCL would kill the chronic myeloid leukemia U937 (expressing mutant p53) and the multiple myeloma MM1.S (expressing wild-type p53) cell lines in which the

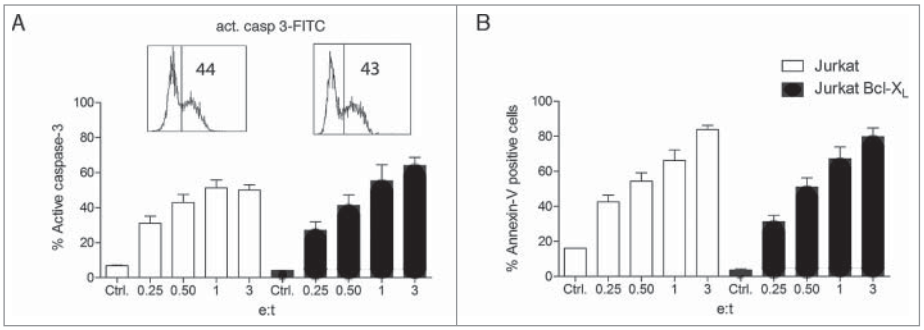

Figure 5. Activated NK cells induce caspase-3 activation in target cells overexpressing Bcl-X_L and kill mutant Jurkat cells in the presence of a caspase inhibitor. Natural killer (NK) cells were enriched by magnetic isolation (MACS) from peripheral blood mononuclear cells (PBMCs) activated during 5 days stimulation with Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs). Activated NK cells were incubated with Jurkat Neo (control) or with Jurkat.Bcl-X_L cells for 4 hours at different effector/target ratios. Caspase-3 activation (A) or phosphatidyl-serine (PS) externalization on the plasma membrane Annexin-V staining (B) was analyzed by staining and 3-color fluorescence cytometry. Results are presented as mean ±SEM of 4 independent donors. Insets show a representative histogram analysis of caspase 3 activation. Numbers correspond to the percentage of cells as gated by the vertical bars.

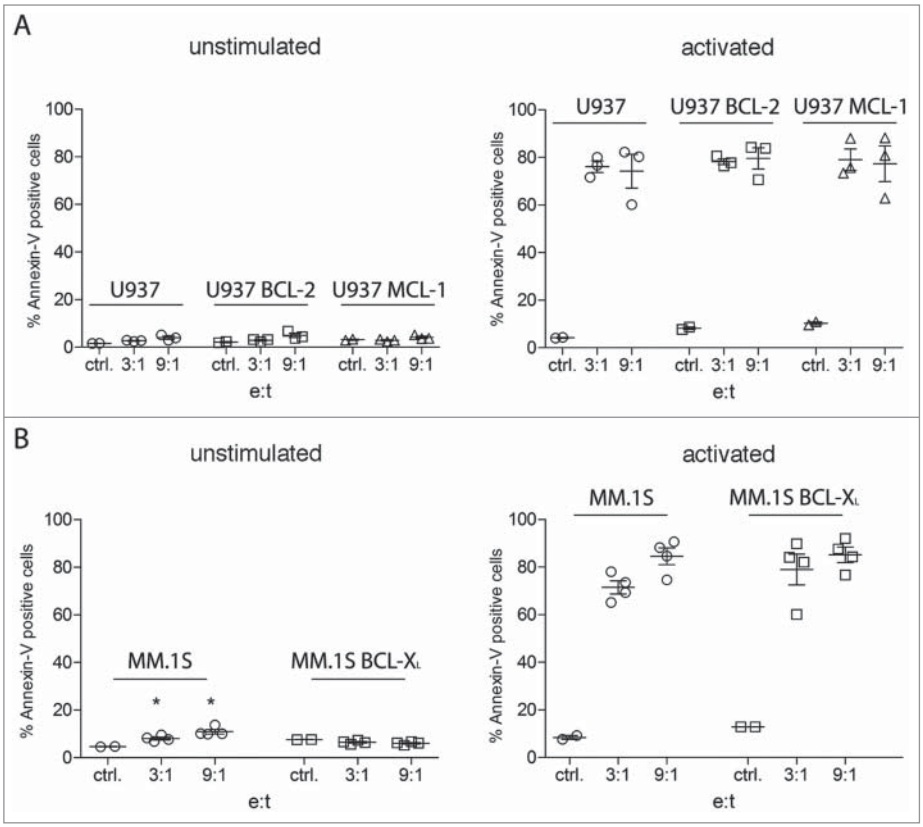

Figure 6. R69 protocol-activated NK cells kill chemotherapy-resistant mutant cell lines from other types of haematological tumors. Natural killer (NK) cells were enriched by magnetic isolation (MACS) from freshly isolated peripheral blood mononuclear cells (PBMCs) or from PBMCs activated during 5 days with Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs). NK cells were then incubated with U937 (A) and with MM1.S (B) cells or with the indicated mutants for 4 hours at different effector/target ratio. Phosphatidyl-serine (PS) exposure on the plasma membrane was detected by Annexin-V staining was analyzed by 3-color fluorescence cytometry. Results are presented as the mean ±SEM of 3–4 independent donors. Each symbol corresponds to an independent NK cell donor. Statistical analysis was performed by 1-way ANOVA with Bonferroni´s post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

proto-oncogenes Bcl-2, Bcl-X_L or Mcl-1 are overexpressed. As shown in **Figure 6** unstimulated NK cells only induced low or undetectable levels of cell death of MM1.S and U937 parental cells, respectively. As in the case of Jurkat cells, overexpression of Bcl-X_L prevented cell death induced by freshly isolated NK cells in MM1.S cells. R69-LCL activated NK cells were able to kill both U937 (**Fig. 6A**) and MM1.S (**Fig. 6B**) parental cells. Overexpression of the anti-apoptotic proteins Bcl-2 or Mcl-1 in U937 cells (**Fig. 6A**) or Bcl-X_L in MM1.S cells (**Fig. 6B**) did not confer protection from cell death induced by activated NK cells. These results indicate that R69-LCL activated human NK cells are capable of killing cancer cell lines of different haematopoietic lineages despite mutations typically conferring apoptotic resistance.

NK cells activated with R69-LCL selectively kill cells from patients with B cell chronic lymphocytic leukemia

To analyze the therapeutic potential of our findings we next tested whether R69-activated NK cells could also kill primary tumor cells derived from patients with B cell chronic lymphocytic leukemia (B-CLL). In parallel, we tested if activated NK cells showed selectivity against transformed cells by analyzing their cytotoxic potential against peripheral blood lymphocytes (PBLs) from healthy donors. As shown in **Figure 7**, R69-LCL activated NK cells were able to kill cells from B-CLL patients although at a variable degree, which generally oscillated between 20 and 60% after 4 hours incubation with the R69-LCL activated NK cells. Spontaneous death of CLL cells was always lower than 20% (data not shown). In contrast freshly isolated NK cells did not show cytotoxicity against CLL cells (data not shown). All patients selected for this study showed a percentage of $CD19^+CD5^+$cells higher than 80%. When $CD19^+$ MACS enriched CLL cells were used as target cells we found similar results (data not shown). PBLs were almost completely resistant to activated NK cells. Importantly, we show that R69-LCL activated NK cells killed

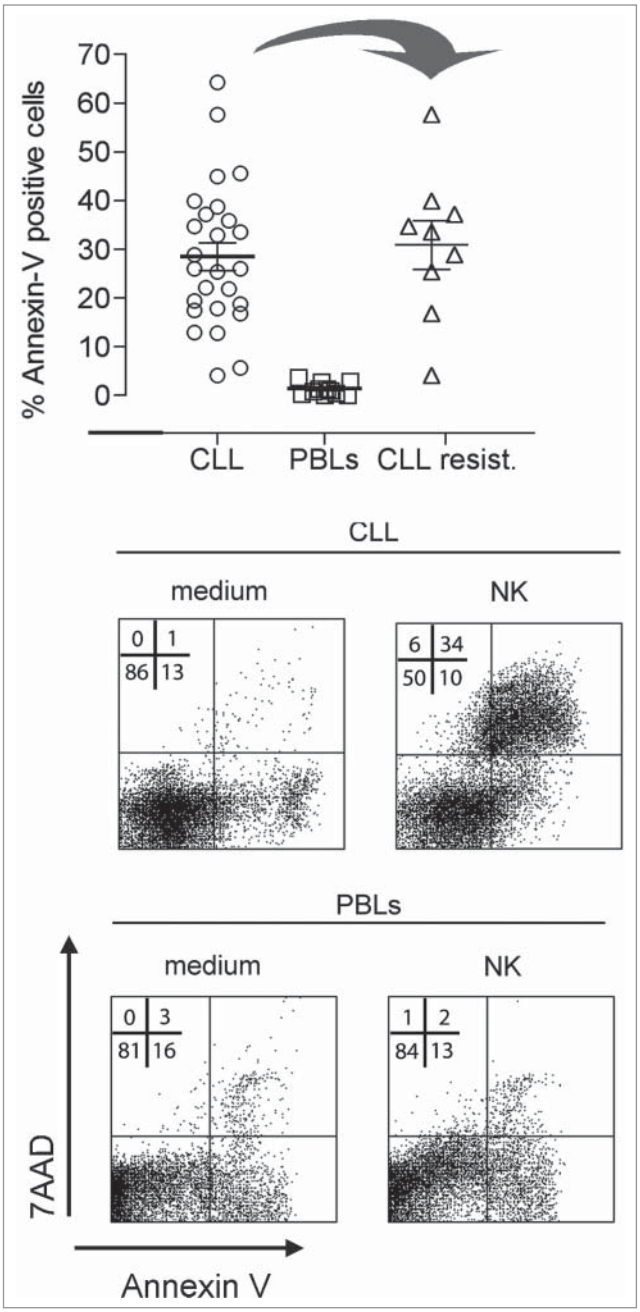

Figure 7. Activated NK cells kill patient-derived B cell chronic lymphocytic leukemia cells but not lymphocytes from healthy donors. Natural killer (NK) cells were enriched by magnetic isolation (MACS) from peripheral blood mononuclear cells (PBMCs) activated during 5 days stimulation with Epstein-Barr virus positive lymphoblastoid B cells (R69-LCLs). Subsequently, they were incubated with PBMCs from chronic lymphocytic leukemia (CLL) patients or from healthy donors (PBLs) for 4 hours at 9:1 effector/target cell ratio. Phosphatidyl-serine (PS) exposure on plasma membrane was detected by Annexin-V staining and membrane permeabilization was detected by uptake of 7-aminoactinomycin-D (7AAD) and analyzed by 3-color fluorescence cytometry. A cohort of 14 CLL patients, 8 PBLs donors and 18 NK cell donors was used in these analyses. Data in the graphics correspond to the combination of different NK cell donors with cells from different CLL patients or healthy donors. Top graphic also includes the cytotoxicity of different NK cells donors on B-CLL cells from 4 patients who presented resistance to chemotherapy (B-CLL Resist). Results are presented as the the mean ±SEM.

leukemia cells derived from patients with a clinical history of resistance to chemotherapy.

Discussion

Allogeneic NK cells are emerging as one of the most promising tools in cell immunotherapy to treat haematological malignancies with poor prognosis.[23,24,44] Here, we show that NK cells activated with an EBV$^+$ lymphoblastoid cell line (R69-LCL) efficiently kill a variety of haematological cancer cell lines and primary tumor cells from patients with B-CLL. R69-LCL NK cell activation, which does not depend on exogenously added cytokines, is also effective in generating NK cells that kill haematological cancer cell lines with a multidrug resistance phenotype acquired by specific mutations that block the apoptotic machinery, as well as chemotherapy resistant B-CLL cells.

LCLs have been widely used as accessory cells to induce the proliferation and activation of NK cells in the presence of exogenous cytokines.[29,45] During the last years the HLA-I deficient leukemic cell line K562 has been introduced as a stimulatory cell supporting NK cell activation and expansion *in vitro* using cytokines such as IL2, IL15, or IL21.[28,32,33] However, our data indicate that NK cells activated with R69-LCL kill haematological cancer cell lines more efficiently than those activated with K562 and IL2 or with K562 in combination with IL2 and IL15. This effect is not restricted to R69 cells since another LCL line, 721.221, stimulates NK cell cytotoxicity to a similar extend, which suggest that, in general, the stimulatory potential of LCLs is higher than that of K562 cells. We do not yet have a full explanation for this effect, although our data suggest that this is likely independent of HLA-I expression levels in stimulating cells as R69 cells express high levels of membrane HLA-I and all known epitopes for inhibitory KIRs, meanwhile 721.221 cells do not express HLA molecules ([35,36] and **Fig. S2**). In line with this finding we have previously found that genes related to NK cell activation and cytotoxicity increased in R69-stimulated cells compared to K562-stimulated cells, *e.g.* granzyme B and IL12 and IL2 receptors.[30] In addition, we have observed here some changes in expression of specific NKRs (**Fig. 2**), Although the involvement of these receptors has not been characterized in detail, we have observed that the physical interaction of LFA-1 with ICAM-1 is required for NK cell cytotoxicity considering a soluble form of ICAM-1 is inhibitory.[38]

Regardless, our data clearly show that R69-activated NK cells are capable of killing B cell lymphoma Raji or cells from B-CLL patients, which are traditionally reported as resistant to NK cells,[46,47] as well as cells with mutations that confer chemoresistance. The increase in the cytotoxic potential of NK cells mainly correlates with the increase in the amount of intracellular GzmB, meanwhile Perf, GzmA GzmK and GNLY expression remains unaltered. These data are in agreement with previous findings showing that NK cell cytotoxic activity correlates with GzmB expression and that inhibition of GzmB via synthetic inhibitors impairs NK cell-mediated cell death.[42,48]

The potential ability of activated NK cells to efficiently kill a variety of cell lines lacking expression of pro-apoptotic genes or overexpressing their anti-apoptotic counterparts merits special attention, being the major highlight of the work presented herein. The relevance of our findings is supported by results showing that leukemia cells isolated from chemotherapy-resistant B-CLL patients are readily killed by activated NK cells. Specifically, it should be noted that our data indicate that activation of NK cells is a prerequisite to kill target cells expressing the indicated mutations. This is of clinical relevance, particularly since most of the previous clinical works have used unstimulated allogeneic NK cells.[21,25-27,49,50] In addition, the selected activation protocol is not trivial, since our present and previous [30] data support the notion that encounter with various target cells significantly changes the activation pattern and, thus, the potency of NK cell-mediated cytotoxicity. In summary our results show that NK cells can be prone to a particular activated state capable of eliminating chemoresistant tumor cells.

Over the course of recent years, great efforts have ensued to develop novel drugs targeting the anti-apoptotic genes of the Bcl-2 family, such as ABT-737 and ABT-199, agents that have shown modest efficacy against various haematological cancers.[51-53] However, these inhibitors present certain limitations, since they do not equally inhibit the anti-apoptotic proteins Bcl-2 and Bcl-X_L, are ineffective against Mcl-1, and further have been shown to provoke thrombocytopenia (ABT-737).[51-54] Our data show that certain activated NK cells can overcome Bcl-2, Bcl-X_L or Mcl-1 overexpression, or conversely, the loss of their pro-apoptotic counterparts, namely Bak and Bax or Bim, or even the absence of the guardian of the genome, p53. Taken together, our findings suggests that this therapy might be more effective than BH3 mimetics in regards to treating tumors exhibiting a disrupted balance between pro- and anti-apoptotic proteins of the Bcl-2 family, even in the absence of caspase activity.

Despite the high cytotoxic potential of NK cells activated with R69-LCL, these cells still present selectivity against transformed cells. R69-LCL-activated NK cells efficiently kill B-CLL cells but present no activity against PBLs from healthy donors. This finding, which could be explained by the absence of stress ligands in normal cells, is supported by previous works showing that treatment with allogeneic NK cells is safe and present neither secondary toxicity nor induce graft-versus-host-disease (GVHD) against healthy cells and tissues.[27,49,55]

In summary, our findings are among the first to indicate that activation of human NK cells is sufficient to overcome multidrug resistance due to mutations in the apoptotic machinery occurring in haematological neoplasias. This activation does not require the addition of exogenous cytokines, correlates with an increase in the intracellular expression of granzyme B and correlates with effective killing of leukemic cells from B-CLL patients. The clinical relevance of our findings is clear, particularly within the context of allogeneic NK cells administered to treat haematological malignancies with poor prognosis, and provide a molecular rationale for the successful use of allogeneic NK cells in the treatment of patients with refractory multiple myeloma or acute myeloid leukemia. Moreover, our results encourage the use of specific therapeutic regimens that stimulate NK cell activation rather than unstimulated NK cells for the immunotherapeutic treatment of highly chemoresistant neoplasias.

Materials and Methods

Patient samples and cell lines

The human acute T leukemia cell line Jurkat, the MHC-I deficient chronic myeloid leukemia cell line K562, the Burkitt´s lymphoma cell line Raji and the pro-monocytic leukemia cell line U937 were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), antibiotics and Glutamax. The different transfectants of these cell lines are described in detail in Supplementary Materials and Methods.

The EBV transformed B lymphoblastoid cell line (LCL) R69 have been previously described.[30]

All cells were tested for mycoplasm contamination by PCR.

Blood samples from patients with Chronic Lymphocytic Leukemia (CLL) were obtained from Hospital Clinico Lozano Blesa de Zaragoza. They were processed by Ficoll gradient centrifugation to obtain peripheral blood mononuclear cells (PBMCs) and stored frozen in liquid nitrogen until their use. In all cases the percentage of $CD19^+CD5^+$ cells was higher than 80% and no differences were observed between frozen and fresh CLL cells regarding their susceptibility to NK cells. This study was approved by Ethics Committee for Clinical Research of Aragon (CEICA), number: C.I.PI11/006.

Antibodies, reagents, and flow cytometry

Cell populations were analyzed for cell surface marker expression by cytofluorimetric analysis. The following fluorophore-conjugated antibodies were used: CD56-PE, CD56-FITC, CD56-APC, CD3-PE, CD3-FITC, CD16-APC and CD16-FITC all from Miltenyi Biotec; CD14-FITC from BD PharMingen and CD4-FITC from Invitrogen. The HLA-E-PE (clone 3D12) and HLA-ABC-FITC (clone W6/32) antibodies were from eBioscience. The NKR antibodies (all PE conjugated) were: NKp30 (CD337; clone az20), NKp44 (CD336; clone Z231), NKp46 (CD335; clone Bab281), NKG2A (CD159a; clone Z199), ILT2 (CD85j; clone HP-F1) from Beckman Coulter; NKG2D (CD314; clone 1D11), KIR2DL2/DL3/S2 (CD158b; clone CH-L), KIR3DL1 (CD158e1, NKB1; clone DX9) from BD PharMingen; KIR2DL5 (CD158f; clone UP-R1) from BioLegend; KIR2DL1/DS1 (CD158a/h; clone 11PB6) from Miltenyi Biotec; DNAM-1 (CD226; clone 102511) and NKG2C (CD159c; clone 134591) from R&D Systems. All antibodies were diluted in PBS with 5% FCS and 0.1% Sodium Azide for cell staining. Intracellular expression of perforin (Perf), granzymes (Gzms) and granulysin (GNLY) were analyzed by FACS using the following antibodies: GzmA-FITC, GzmB-PE, perforin-FITC from BD PharMingen; GzmK-FITC from Santa Cruz Biotechnology; and GNLY-PE from eBiosciences.

Isolation and activation of human NK cells

Human *ex vivo* NK cells were enriched by using anti-CD56 MicroBeads with a MultiStand MACS (MACS, Miltenyi Biotec) from freshly isolated PBMCs.

Activation of human primary NK cells was pursued by culturing PBMCs for 5 days under 2 different conditions: 50 UI/mL rIL2 alone or in combination with 10 ng/mL rIL-15 together with Mitomycin C-inactivated K562 cells or Mitomycin C-treated R69- or 721.221-LCL without rIL2 at 10:1 PBMC:Stimulator ratio. Subsequently, NK cells were enriched by using anti-CD56 MicroBeads with a MultiStand MACS (MACS, Miltenyi Biotec).

PBMCs were obtained from healthy donors (Blood and Tissue Bank of Aragon; Approved by the CEICA, number: C.I. PI11/006). NK cell purity ($CD56^+$/CD3-) was higher than 90% in all cases.

In some cases the absolute numbers of NK cells ($CD56^+$CD3-) in PBMCs cultures was quantified by using BD Trucount™ Tubes (BD Biosciences) following manufacturer's instructions.

Analyses of cell death by flow cytometry

NK cells were labeled with 1 μM of CellTracker™ Green CMFDA or CellTracker™ Violet BMQC (Life Technologies) and incubated with target cells at different E:T cell ratios for 4 hours. In order to inhibit granule exocytosis, the calcium chelating agent ethylene glycol tetraacetic acid (EGTA) was added to the cell cultures in the presence of $MgCl_2$. In order to inhibit the effector caspases (caspase-3/-7), Z-DEVD-fmk (Bachem) was used at concentration of 100 μM. Subsequently, Phosphatidylserine (PS) translocation and membrane damage and caspase-3 activation were analyzed in the green or violet fluorescence negative target cell population by flow cytometry, as previously described.[12]

Statistical analysis

The statistical analysis of the difference between means of samples was performed using the Students t test, one-way or 2-way ANOVA test depending on the properties of the experimental groups. The results are given as the confidence interval (p). All the experiments described in the figures were performed at least 3 times with similar results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental data for this manuscript can be accessed on the publisher's website.

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