Recognition of adult and pediatric acute lymphoblastic leukemia blasts by natural killer cells

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ABSTRACT

In this study, we aimed to investigate the pathways of recognition of acute lymphoblastic leukemia blasts by natural killer cells and to verify whether differences in natural killer cell activating receptor ligand expression among groups defined by age of patients, or presence of cytogenetic/molecular aberrations correlate with the susceptibility to recognition and killing. We analyzed 103 newly diagnosed acute lymphoblastic leukemia patients: 46 adults and 57 children. Pediatric blasts showed a significantly higher expression of Nec-2 (P=0.03), ULBP-1 (P=0.01) and ULBP-3 (P=0.04) compared to adult cells. The differential expression of these ligands between adults and children was confined to B-lineage acute lymphoblastic leukemia with no known molecular alterations. Within molecularly defined subgroups of patients, a high surface expression of NKG2D and DNAM1 ligands was found on BCR-ABL$^+$ blasts, regardless of patient age. Accordingly, BCR-ABL$^+$ blasts proved to be significantly more susceptible to natural killer-dependent lysis than B-lineage blasts without molecular aberrations (P=0.03). Cytotoxic tests performed in the presence of neutralizing antibodies indicated a pathway of acute lymphoblastic leukemia cell recognition in the setting of the Nec-2/DNAM-1 interaction. These data provide a biological explanation of the different roles played by alloreactive natural killer cells in pediatric *versus* adult acute lymphoblastic leukemia and suggest that new natural killer-based strategies targeting specific subgroups of patients, particularly those BCR-ABL$^+$, are worth pursuing further.

Introduction

The clinical management of acute lymphoblastic leukemia (ALL) patients has witnessed major changes over the years. This has translated into progressive improvements in the prognosis of childhood ALL, with current 5-year survival rates of more than 80%,[1] while the therapeutic advancements in adult ALL have been more limited.

The main cause of treatment failure, particularly in the adult setting, is still represented by the high rate of relapse following the achievement of complete remission (CR).[2] Most patients reach CR but still have evidence of persistent minimal residual disease (MRD) after induction and consolidation, and tend to relapse with time.[3,4] Efforts have been made to develop therapeutic procedures aimed at controlling/eradicating MRD.[5,6]

Over the years, the anti-leukemic potential of natural killer (NK) cells and their ability to regulate normal and possibly neoplastic hematopoietic precursors has raised considerable interest.[7-9] NK clones of donor origin with killing capabilities against recipients' leukemic cells have been shown to emerge in the post-transplantation period after HLA-mismatched hematopoietic stem cell transplantation (HSCT).[10-13] In adult patients, it has been reported that most acute myeloid leukemia (AML),[14] but only a minority of ALL cells, are susceptible to NK-cell mediated lysis.[15,16] On the contrary, recent studies of high-risk pediatric ALL undergoing haploidentical HSCT have highlighted the importance of choosing donors with alloreactive NK cells[17,18] to successfully cure these patients. The biological reasons responsible for the different susceptibility of adult and pediatric ALL blasts to the lytic effect played by alloreactive NK cells are so far still unknown.

NK cell recognition and killing capabilities are finely regulated by the activity of multiple receptors with either activating or inhibitory functions.[19-21] An array of different HLA class-I-specific inhibitory receptors, termed "killer cell immunoglobulin-like receptors" (KIR), has been identified; these receptors recognize HLA class I allele groups (KIR ligands) and share the function of preventing the killing of normal major histocompatibility complex (MHC) class-I-positive autologous cells.[10] In the absence of efficient inhibitory interactions, target cells may be susceptible to NK cell-mediated killing.[22,23] Recently, a role for KIR with activating functions in the recognition of hematopoietic malignancies has been shown.[24,25] In humans, the major non-MHC class-I-receptors responsible for tumor recognition by NK cells are the natural cytotoxicity receptors (NCR: NKp30, NKp44 and NKp46),[26] NKG2D[27,28] and DNAM-1.[29] While some of these receptors are still orphan of their ligands, MICA/B and ULBPs have been discovered to be the ligands for NKG2D,[30] whereas the Poliovirus receptor (PVR, CD155) and Nectin-2 (Nec-2, CD112) interact with DNAM-1.[31,32] The NK cell-mediated lysis of tumor cells involves several such receptors, depending on the type of malignancy. It has been described, for example, that recognition and lysis of AML blasts occurs mainly through the NCR and DNAM-1 receptors.[33] On the contrary,

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the pathways of NK cell/ALL blast interaction still need to be better clarified.

In this study, we addressed the pathways of NK cell/ALL blast recognition and compared the expression of activating ligands among pediatric and adult ALL patients. Molecularly-defined subgroups of ALL patients were considered in the analysis, and functional tests were performed to confirm the results of the phenotypic findings.

Methods

Patients

A total of 103 newly diagnosed cases of ALL, including 46 adults (median age 34 years; range 18-74 years) and 57 children (median age 4 years; range 0.1-17 years) were investigated between December 2011 and February 2013.

Based on the immunophenotypic profile, the case series was subdivided as follows: 90 B-lineage ALL (B-ALL) (39 adult and 51 pediatric cases) and 13 T-lineage ALL (T-ALL) (7 adult and 6 pediatric cases). Within the adult B-ALL cohort, the following fusion transcripts were identified: *BCR-ABL* in 15 cases, *MLL-AF4* in 7, *E2A-PBX1* in 2, while the remaining 15 cases were negative for the most common molecular lesions found in ALL. In pediatric B-ALL, the BCR-ABL transcript was found in 6 patients, *MLL-AF4* in 3, *MLL-ENL* in 1, *TEL-AML1* in 10; 31 patients did not show any of the molecular aberrations investigated. Informed consent for biological studies was obtained from patients or their legal guardians in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee.

Immunofluorescence and flow cytometry

Phenotypic analyses were performed on a FACSCanto flow cytometer using the FACSDiva software (BD Bioscience, San Jose, CA, USA). Evaluation of the NKG2D and DNAM-1 ligands on primary ALL blast cells was performed using different combinations of the following monoclonal antibodies (mAbs): anti-HLA-ABC (BD Biosciences); anti-PVR (AbD Serotec, Oxford, UK); anti-Nec2 (BD Biosciences); anti-ULBP-1, anti-ULBP-2, anti-ULBP-3, anti-MIC-A and anti-MIC-B (RnDSystems, Minneapolis, MN, USA). The expression of NKG2D and DNAM-1 activating receptors on NK cells was analyzed using anti-CD16, anti-CD56, anti-CD3 (BD Biosciences), anti-NKG2D (R&D System) and anti-DNAM1 (AbD Serotec) mAbs.

Natural killer cell isolation and culture

For NK-cell enrichment, a 2-step immunomagnetic procedure was used, consisting of a $CD3^+$ T-cell depletion followed by a $CD56^+$ cell positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany). For *ex vivo* cell expansion, isolated NK cells (1×10^5/mL) were suspended in Cellgro SCGM serum-free medium (CellGenix, Freiburg, Germany) supplemented with 5% human serum Type AB (Li StarFish, Milan, Italy), 500 U/mL Interleukin (IL)-2 (Proleukin, Chiron, Amsterdam, The Netherlands) and 50 ng/mL IL-15 (CellGenix) in the presence of irradiated (35 Gy) autologous monocytes, T and B cells as feeder (2.5×10^5/mL), and cultured for 14 days at 37°C. IL-2 and IL-15 were also added to the culture medium during the last 24 h of the expansion period. Only good manufacturing practice (GMP) and clinical grade materials were used.

Cytotoxicity assay

Cytotoxic activity of expanded NK cells against the erythroleukemia cell line K562 and against primary adult and pediatric ALL blasts was determined in a standard 4-h ^{51}Chromium (^{51}Cr) release assay. NK effector:target (E:T) cell ratios ranged from 50:1 to 0.39:1, using $2x10^5$ target cells in triplicate wells. For blocking experiments, NK cells were treated with the anti-NKG2D (R&D System) or anti-DNAM1 (AbD Serotec) neutralizing mAbs prior to co-culture with target ALL cells in the cytotoxic assay; the anti-CD56 (C218) mAb (Beckman Coulter GmbH, Munich, Germany) was used as control.

Statistical analysis

Statistical analysis was performed using Student's paired t-test. $P<0.05$ was considered statistically significant.

Results

Expression of NKG2D and DNAM1 ligands on ALL blasts

In order to assess ALL susceptibility to NK cell-mediated lysis, the expression pattern of the ligands for NK cell activating receptors NKG2D and DNAM1 was investigated on primary adult and pediatric leukemia cells. These include MIC-A/B and ULBP1-3 among NKG2D ligands, and Nec-2 and PVR DNAM1 ligands.

As shown in Table 1, ULBP-1/3, MIC-B and Nec-2 were the most highly and frequently expressed ligands in both cohorts of patients. Interestingly, the expression of Nec-2 and PVR on the one hand and of MIC-A and MIC-B on the other was inversely correlated both for adult ($P<0.0001$ and $P=0.0008$, respectively) and pediatric ($P<0.0001$ and $P=0.0005$, respectively) cases, Nec-2 and MIC-B being more expressed than PVR and MIC-A.

The phenotypic analysis performed within molecularly defined subgroups of adult ALL revealed that cells carrying the *BCR-ABL* fusion gene presented an overall high surface expression of ligands for NKG2D and DNAM1 (Figure 1A). In particular, when compared to ALL carrying no known molecular markers, *BCR-ABL*$^+$ cases showed significantly higher levels of ULBP-1, ULBP-3 and MIC-B ($P=0.0084$, $P=0.026$ and $P=0.033$, respectively), while *MLL-AF4*$^+$ B-ALL and T-ALL cases displayed a higher density only of ULBP-1 ($P=0.0013$ and $P=0.034$, respectively). Finally, *BCR-ABL*$^+$ ALL and T-ALL cells displayed a higher, though not statistically significant, MFI of expression of HLA class-I surface molecules (A, B, C) (*data not shown*).

Unlike adult ALL, the comparison of NK activating receptor ligand expression levels in the pediatric cohort did not reveal any difference when ALL cases were subdivided according to both cell lineage and presence of molecular aberrations (Figure 1B). Furthermore, no significant differences in HLA-class-I surface density were observed (*data not shown*). These findings suggest a comparable susceptibility of the whole pediatric ALL cohort to NK cell recognition and lysis.

Additional differences were observed when adult and pediatric patients were compared. In fact, blasts of pediatric patients showed an increased expression of Nec-2 ($P=0.03$), ULBP-1 ($P=0.015$) and ULBP-3 ($P=0.04$) ligands compared to adult cases. The analysis was then performed within lineage- and molecularly-defined subgroups of patients. T-ALL of both patient cohorts showed a similar expression of the above-mentioned ligands. On the contrary, when comparing adult and pediatric B-ALL, in the pediatric group a significantly increased expression of Nec-2 ($P=0.033$), ULBP-1 ($P=0.016$) and ULBP-3 ($P=0.045$) was evident (Figure 2A). In addition, blasts from pediatric patients without molecular aberrations showed a significant increase of ULBP-1/3

when compared to adult cases (P=0.0014 and P=0.005, respectively) (Figure 2B). Finally, no differences were observed in *BCR-ABL*$^+$ and *MLL-AF4*$^+$ B-ALL cells between adult and pediatric patients, as NKG2D and DNAM-1 ligands were strongly expressed in both groups. Taken together, these results suggest that adult *BCR-ABL*$^+$ and *MLL-AF4*$^+$ B-ALL cells are as susceptible to NK-mediated lysis as the entire group of pediatric ALL samples.

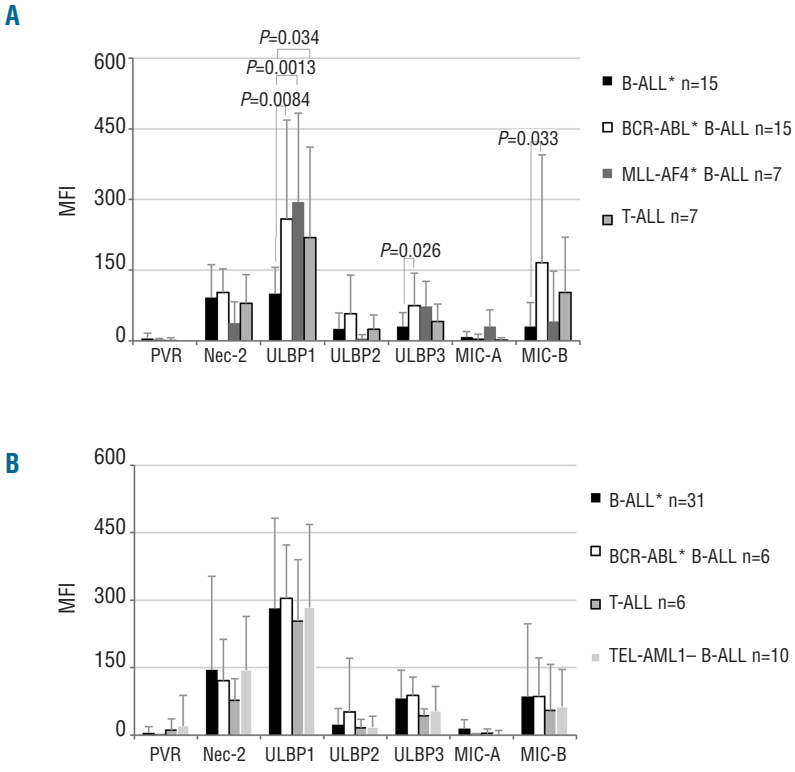

Figure 1. Phenotypic analysis of NKG2D and DNAM-1 ligands in adult (A) and pediatric (B) ALL patients, according to cell lineage and presence of molecular aberrations. Results are expressed as mean MFI±SD. MFI was calculated considering the MFI of the relevant mAb respect to the MFI of its relative isotype control. *B-ALL without molecular aberrations.

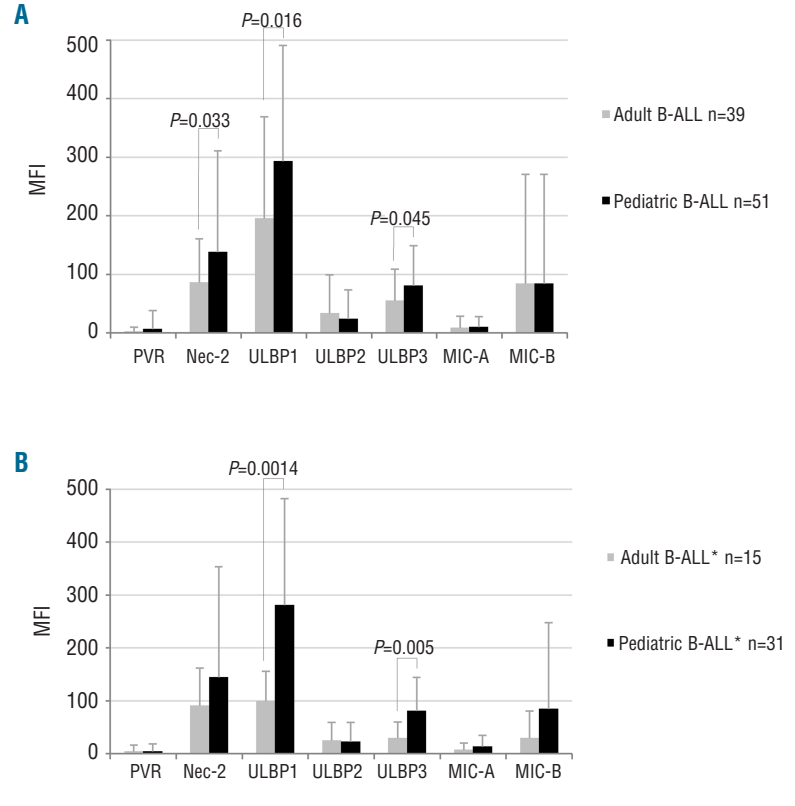

Figure 2. NKG2D and DNAM-1 ligand expression in adult and pediatric B-ALL. Comparison of activating receptor ligand surface levels between adult and pediatric B-ALL (A) and adult and pediatric B-ALL without molecular markers (B). Results are expressed as mean MFI±SD. * B-ALL without molecular aberrations.

NK-mediated cytolysis in adult and pediatric ALL

Characteristics of the NK effector cell population

In order to evaluate whether the differences found in NKG2D and DNAM-1 ligand expression were associated to a corresponding variability in NK cell lysis of ALL cells, NK cells from healthy donors were purified and expanded *ex vivo* in the presence of IL-2, IL-15 and autologous feeder cells.

Using a 2-step immunomagnetic selection, we obtained an average of 98.4%±1.4% CD56$^+$ cells. The percentage of contaminating T lymphocytes after depletion was 0.4%±0.7%, while that of NK T lymphocytes (CD56$^+$CD3$^+$CD16$^-$) was 4.6%±8.4%. At the end of the culture period, NK cells presented a mean expansion fold of 39.5 times. The phenotypic analysis performed on the expanded NK cell population showed an increase of CD56$^+$ NK lymphocytes of up to 99.3%±0.5%, in association with a decrease in T (0.01%±0.03%) and NK T (2.4%±4.9%) contaminating lymphocytes. These expanded cells displayed a high expression of both CD56 and CD16 antigens (*data not shown*). The expression of NKG2D and DNAM-1 activating receptors was also evaluated on NK cells both before and after expansion. As shown in Figure 3, both activating receptors presented a significantly increased expression after *in vitro* expansion (NKG2D MFI: 109.1±94.3 *vs.* 1417.2±962.3, *P*=0.0004; DNAM-1 MFI: 902.1±225.9 *vs.* 3266.1±1292.4, *P*=0.0007).

NK cell cytotoxic activity against primary ALL blasts

Expanded NK cells from 10 different healthy donors

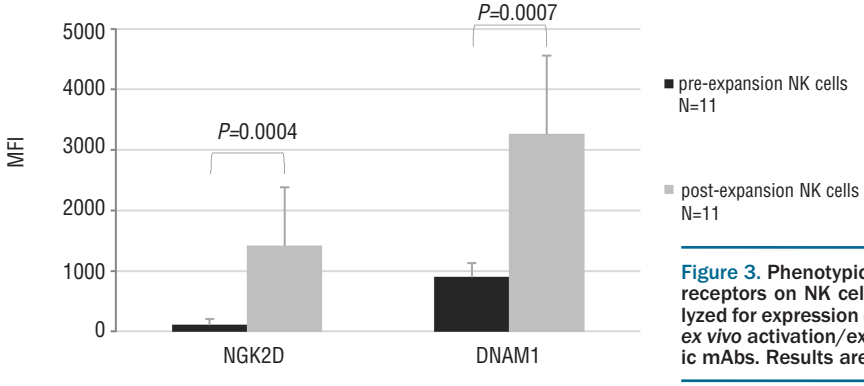

Figure 3. Phenotypic analysis of NKG2D and DNAM-1 activating receptors on NK cells from healthy donors. NK cells were analyzed for expression of NKG2D and DNAM-1, before and after NK *ex vivo* activation/expansion, by incubating cells with the specific mAbs. Results are expressed as mean MFI±SD.

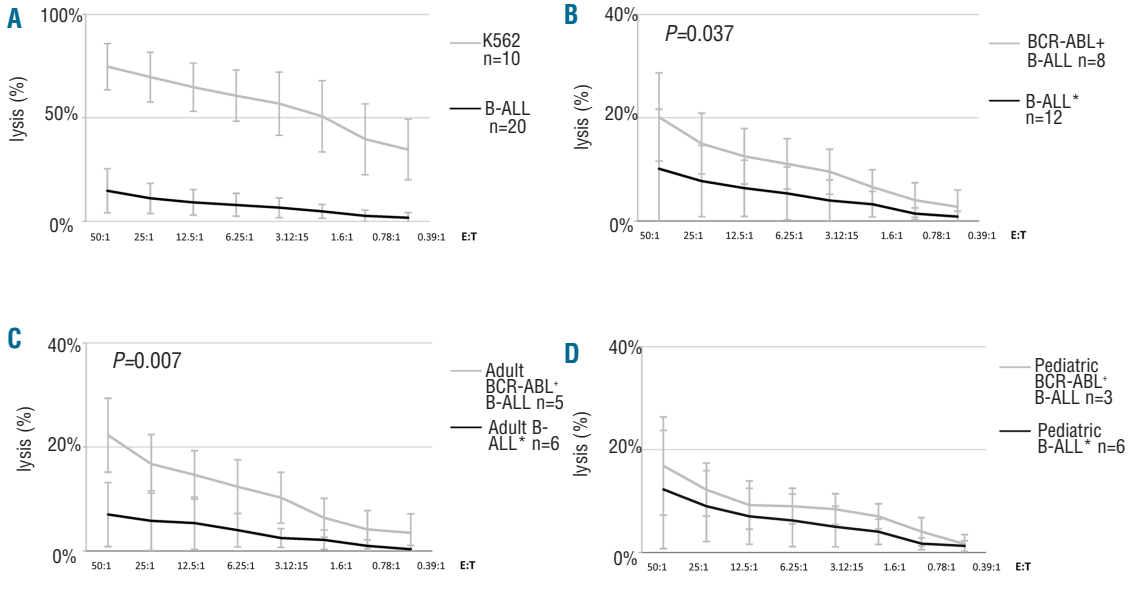

Figure 4. NK cytolytic activity against ALL blasts. NK-mediated lysis of K562 cells and B-ALL primary blasts (A). Comparison of NK-mediated lysis between BCR-ABL$^+$ and molecularly negative B-ALL (B). Comparison of NK-mediated lysis between BCR-ABL$^+$ adult ALL and molecularly negative adult B-ALL (C). Comparison of NK-mediated lysis between BCR-ABL$^+$ pediatric B-ALL and molecularly negative pediatric B-ALL (D). In these cytolytic assays, ^{51}Cr-labeled K562, pediatric ALL and adult ALL cells were used as target; alloreactive polyclonal NK cells from healthy donors were used as effectors. Data are expressed as percentage of lysis. *B-ALL without molecular aberrations.

mediated an efficient lysis of K562 target cells (n=10; mean cytotoxicity at a 50:1 E:T ratio: 75.6%±10.7%) and of primary allogeneic B-ALL blasts (n=20; mean cytotoxicity at a 50:1 E:T ratio 14.8%±10.6%) (Figure 4A).

When analyzing NK cell cytotoxic activity towards different subgroups of B-ALL patients, it was found that BCR-ABL$^+$ cases presented a significantly higher susceptibility to NK cell killing activity than molecularly negative samples (P=0.037) (Figure 4B). This difference was much more evident when the analysis was performed among adult cases (*BCR-ABL$^+$ vs.* molecularly negative samples, P=0.007) (Figure 4C), while it was not observed among pediatric cases (*BCR-ABL$^+$ vs.* molecularly negative samples) (Figure 4D).

These results clearly support the presence of a correlation between the intensity of the NK-receptor ligand expression on lymphoid leukemic cells and their susceptibility to NK-mediated killing.

Unlike expanded and activated effectors, freshly selected, unmanipulated NK cells were not capable of exerting any cytotoxic activity against primary ALL blast cells (*data not shown*).

We finally analyzed the relative role of NKG2D and DNAM-1 activating receptors in the induction of B-ALL lysis by performing the cytotoxic assays in the presence of anti-NKG2D or anti-DNAM-1 neutralizing mAbs. When the assay was carried out with the anti-DNAM-1 mAb, the cytotoxic potential of *ex vivo*-generated allogeneic NK cells was significantly inhibited (n=11, mean cytotoxicity at 50:1 E:T ratio from 17.6%±10% to 6.4%±4%, P=0.0067) (Figure 5). On the contrary, the use of the anti-NKG2D mAb revealed a mean percentage of lysis similar to that obtained with the control mAb. These results indicate that the Nec-2/DNAM-1 interaction plays a more crucial role in NK cell mediated killing of leukemia blasts than that involving ULBP1-2-3/NKG2D or MICA-B/NKG2D.

Discussion

The results of this study indicate that adult and pediatric ALL blasts show a different expression of the known ligands for NK cell activating receptors. In fact, blasts of pediatric origin have an increased expression of Nec-2, ULBP1 and ULBP3 compared to adult cases. This difference is particularly evident considering the subset of B-ALL blasts carrying no known molecular markers. Specific phenotypic patterns of expression are also associated with molecularly defined subgroups of ALL patients. In particular, when considering Philadelphia-positive (Ph$^+$) ALL patients, the increased intensity of NK cell activating ligand expression that distinguishes this subgroup of patients appears

Table 1. Surface expression of NKG2D and DNAM-1 NK activating receptor ligands in adult and pediatric ALL patients.

	Adult ALL						Pediatric ALL					
	Total cases (n=46)		B-ALL (n=39)		T-ALL (n=7)		Total cases (n=57)		B-ALL (n=51)		T-ALL (n=6)	
	n/N	MFI	n/N	MFI	n/N	MFI	n/N	MFI	n/N	MFI	n/N	MFI
NKG2D ligands												
MIC-A	12/46	8±18.4	11/39	9.1±19.7	1/7	1.8±4.9	22/57	1.8±4.9	21/51	9.9±18.1	1/6	4±9.3
MIC-B	26/46	87±173.7	21/39	84.2±186.9	5/7	102.7±117.1	30/57	72.3±131.6	28/51	74.3±135.3	2/6	54.8±102.8
ULBP-1	46/46	199.4±174	39/39	195.9±173.2	7/7	219.1±191.8	57/57	289.1±191.6	51/51	293.3±174	6/6	253.8±136.6
ULPB-2	28/46	32.3±60.1	22/39	33.7±65.6	5/7	24.4±30.2	34/57	23.2±47.2	33/51	24±49.6	1/6	16.5±19
ULBP-3	44/46	53.1±51.1	37/39	55.3±53.3	6/7	40.9±37.4	54/57	77±65.7	48/51	81.1±68.3	6/6	43.2±14.9
DNAM-1 ligands												
PVR	7/46	2.04±7.1	7/39	2.4±7.74	0/7	-	10/57	7.3±30.8	9/51	6.9±31.4	1/6	10.8±26.5
Nec2	41/46	85.4±71.6	35/39	86.4±73.9	6/7	67±11.3	52/57	131.5±165.2	46/51	137.9±173.1	6/6	67.2±43.3

n/N: positive cases/studied cases; MFI: mean values ± SD.

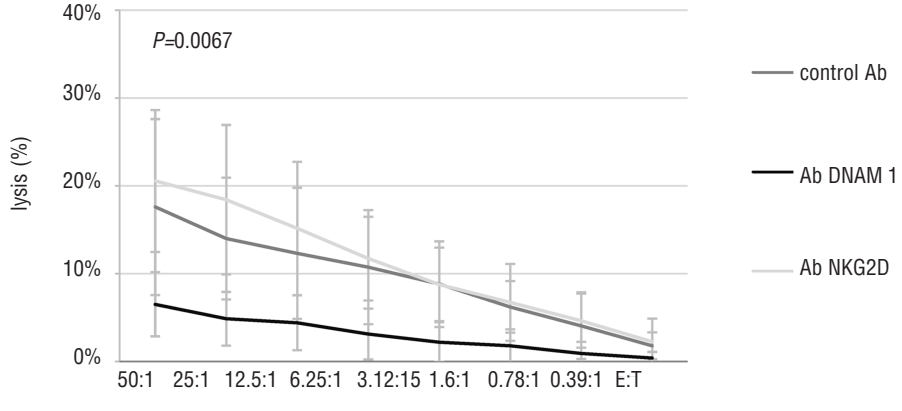

Figure 5. Involvement of NKG2D and DNAM-1 activating NK receptors in ALL cell lysis. For blocking experiments, healthy donor allogeneic NK cells were pre-incubated with anti-NKG2D or anti-DNAM1 neutralizing mAbs, washed and then added to the cytotoxic assay in the presence of ALL target cells. Data are expressed as percentage of lysis.

evident. Cytotoxic tests documented the ability of *in vitro* expanded and activated NK cells of healthy donors to specifically recognize and kill ALL blasts, mainly those carrying the *BCR-ABL* gene fusion and those obtained by pediatric patients. In addition, experiments performed with blocking antibodies identified the Nec-2/DNAM-1 interaction to be the crucial pathway involved in NK cell/ALL blast recognition.

These results are of potential clinical relevance considering that, for the first time, a possible biological explanation of the different roles played by alloreactive NK cells in the pediatric and adult ALL setting has been found. The significantly increased expression in pediatric B-ALL of some of the ligands for NKG2D and DNAM-1, including Nec-2, may explain why the use of an alloreactive donor offers an advantage in terms of prevention of leukemia recurrence.[34] It must be noted that the differential expression of these ligands between adults and children is restricted to B-lineage ALL, while no differences were observed when analyzing T-ALL.

Our study recognized a differential expression of ligands for NK cell activating receptors in molecularly defined subgroups of ALL patients. This is the case of *BCR-ABL*$^+$ ALL blasts, which display, together with the *MLL-AF4*$^+$ subgroup, the highest intensity of ligand expression in the context of adult B-ALL. Importantly, the high expression of ligands for NK cell activating receptors correlated with the degree of susceptibility to lysis by expanded allogeneic NK cells, further supporting the role played by these receptors during the processes of recognition.

The case of Ph$^+$ ALL is of particular interest. The management and prognosis of these patients has changed profoundly following the use of *BCR-ABL*–directed tyrosine kinase inhibitors (TKI) in front-line treatment.[35] Our group has shown that virtually all patients, irrespective of age, can obtain a hematologic CR with the use of 1st and 2nd-generation TKI plus steroids alone, without undergoing systemic chemotherapy.[36,37] Despite these encouraging results, at the end of induction/consolidation most patients show MRD persistence which correlates with a higher likelihood of leukemia relapse.[37,38] The findings reported in the present study, showing the high levels of expression of ligands for NK activating receptors by BCR-ABL$^+$ ALL cells, point to a possible post-CR immunotherapeutic strategy based on the *in vivo* infusion of either autologous or allogeneic activated NK cells with the aim of controlling/eradicating MRD. This possibility is further supported by the results of the cytotoxic tests presented here, confirming the susceptibility of *BCR-ABL*$^+$ ALL cells to NK-mediated killing, and by the previously reported ability of autologous NK cells activated in a non-GMP setting to recognize and kill ALL blasts.[39] Further studies performed in compliance with GMP rules will address this point also in the autologous setting.

The use of blocking antibodies in these functional tests has identified the Nec-2/DNAM-1 interaction to be the pathway of recognition of ALL by NK cells, as already suggested by other authors.[33] As expected, the intensity of Nec-2 and PVR expression was inversely correlated, Nec-2 being expressed in almost 100% of cases and PVR being virtually absent, unlike, for example, superantigen-stimulated T cells which at the cell surface express only PVR with a mechanism involving a DNA-damage response-dependent pathway.[40]

Our methodological approach used for NK cell enrichment and expansion, which is essential to obtain a sufficient number of effectors to pursue a program of adoptive cellular therapy, may be transferred into the clinic for experimental phase I/II studies based on the *in vivo* infusion of *ex vivo* expanded and activated NK cells for the control of MRD in ALL patients. The possible correlation between the intensity of expression of ligands for NK cell activating receptors and the susceptibility to lysis may help identify patients who may gain the most benefit from NK-based immunotherapy and from alloreactive donors in the context of haploidentical HSCT. The relationship between ligand expression and susceptibility to lysis reported here provides a strong biological support to the choice of treatment to suggest for specific cases, thus paving the way to new therapeutic algorithms for a modern management of ALL. This is of particular interest for BCR-ABL$^+$ ALL patients for whom a sequential treatment strategy based on the use of TKI plus steroids as induction treatment, followed by an immune-mediated control of MRD during TKI maintenance, now seems to be worth pursuing, particularly for elderly patients in whom this abnormality is prevalent,[41] and who because of age or comorbidities cannot undergo standard treatments or be considered for transplant programs. This strategy may also take advantage of the previously reported ability of TKIs, particularly dasatinib, of inducing a NK-cell mobilization, activation and proliferation,[42,43] potentially capable of favoring a TKI-induced activation of *ex vivo* expanded and infused NK cells. Further studies should investigate whether TKIs play a role in the expression of NK cell ligands on leukemia cells, therefore possibly modulating the recognition and killing capacity of this population of expanded effectors.

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