Expansion of natural killer cells with lytic activity against autologous blasts from adult and pediatric acute lymphoid leukemia patients in complete hematologic remission

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Background and Objectives. Natural killer (NK) cells constitute an important area of research for hematologic malignancies. The anti-leukemic activity of NK cells against acute myeloid leukemia (AML) blasts has been described, but very few data are available for acute lymphoid leukemia (ALL). The present study was designed to investigate whether: (i) NK effectors could be expanded from adult and pediatric ALL patients in complete remission; (ii) the signal transduction machinery of these cells was preserved; (iii) NK cells showed cytotoxic activity against autologous blasts; (iv) interleukin (IL)-2, IL-12 and IL-15 were able to increase lytic activity in our *in vitro* model; (v) any differences in cytotoxic activity could be found between expanded effectors from adult and pediatric patients.

Design and Methods. We co-cultured patients' peripheral blood mononuclear cells (PBMC) with the feeder cell line RPMI 8866 and analyzed the NK cells' expansion capacity by cell counting and cytofluorimetric analyses. Signal transduction of expanded effector cells was evaluated by Western blot. ^{51}Cr release assays, before and after stimulation with activating cytokines, were performed to analyze the cytotoxic potential of effector cells against tumor cell lines and autologous blast cells. Data were analyzed with t-tests for paired data.

Results. We obtained an average 40-fold increase in NK cells. Signal transduction through the CD16 receptor was preserved. Patients' expanded cells showed cytotoxic activity against target cell lines comparable to that of normal donors. More significantly, these cells also exerted a lytic effect against autologous blasts. In addition, incubating these effectors for 24 hours with IL-2 + IL-15 significantly increased this cytotoxic function. No differences in expansion and cytotoxic activity were found between pediatric and adult patients.

Interpretation and Conclusions. These findings document for the first time the possibility of expanding *ex vivo* cytotoxic effectors with autologous killing capacity from ALL patients in remission, and suggest a new potential immunotherapeutic strategy for the management of early disease recurrence or of residual disease.

Key words: NK, ALL, autologous blasts, cytotoxicity, immunotherapy.

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The anti-leukemic potential of natural killer (NK) cells and their competence in regulating normal and possibly neoplastic hematopoietic precursors have over the years raised considerable interest. The role of NK cells in immunosurveillance against tumor growth is well documented.[1,2] Previous studies have shown that leukemic blasts may be susceptible to the lytic action of lymphokine-activated killer (LAK) cells.[3,4] More recently, NK clones of donor origin have been established in the post-transplant period from HLA-mismatched hematopoietic stem cell transplanted recipients;[5] NK clones were capable of killing recipients' leukemic cells, in the absence of graft-versus-host disease. In addressing the graft-versus-leukemia potential of donor-versus-recipient NK cell alloreactivity, it was found that 100% of acute myeloid leukemia (AML), but only a minority of acute lymphoid leukemia (ALL) cells were killed by alloreactive NK clones. With the goal of optimizing the immunological control of neoplasia, various cytokines have been tested in different *in vitro* and *in vivo* settings. So far, only a few of them have reached clinical use. Interkeukin (IL)-2, IL-12 and IL-15 are cytokines that are active on NK cells. IL-2 and IL-15 are structurally related and have demonstrated overlapping functions, as well as distinct roles in inducing NK proliferation and enhancing cytotoxicity,[6,7] while IL-12 is structurally distinct, has a modest proliferative effect on NK cells, but is capable, alone, of enhancing cytotoxicity.[8] It has been suggested that *in vivo* IL-2 treatment has anti-leukemic potential in AML patients with a limited proportion of residual marrow blasts.[9-11] The role of IL-2-activated NK cells has also been investigated in patients who have undergone an allogeneic bone marrow transplant for chronic myeloid leukemia (CML);[12] in this setting, a clear correlation between the generation of lytic activity against host-derived CML targets

and the risk of relapse has been established. Moreover, activated NK cells have been used to suppress primitive leukemic progenitors from CML patients in long-term autologous cultures, suggesting that autologous IL-2-activated NK cells with potent major histocompatibility complex unrestricted cytotoxic activity are capable of suppressing malignant hematopoiesis.[13]

Different data generated *in vitro* and *in vivo* suggest that IL-15 may play an important role in anti-tumor activity: this citokine induces the expression of mRNA for perforin and granzymes in murine lymphocytes,[14] suppresses the appearance of lung tumor lesions when administered to mice injected with mouse sarcoma cells[15] and prolongs tumor remission induced by cyclophosphamide in rhabdomyosarcoma-bearing mice.[16] More recently, IL-15 has been regarded as a potential co-stimulatory cytokine for the induction of apoptosis in chronic lymphoid leukemia (CLL) cells via the CD40 pathway.[17]

IL-12 is known as NK cell stimulation factor;[18] it enhances NK activity and specific cytotoxic T-cell (CTL) responses,[19] and induces NK and T cells to produce interferon (IFN)α and tumor necrosis factor (TNF)α.[20] These and other effects probably account for the ability of IL-12, alone or in combination with IL-2, to increase lytic activity of peripheral blood mononuclear cells (PBMC) against tumor cell lines[21] and primary allogeneic[22] and autologous[23] leukemic blasts, to correct the defective cytotoxic activity of neoplastic patients at diagnosis[24] and to induce anti-neoplastic activity in murine cancer models.[25]

We have recently demonstrated the possibility of expanding cytotoxic effectors, mainly NK cells, with killing activity against autologous blasts, from AML patients in complete remission (CR).[26] This expanded population of effector cells has an intact signal transduction apparatus and a preserved capacity to produce cytokines important in the cytolytic process. After separation and purification of NK cells from the population of expanded effectors, we demonstrated that most of the lytic effect was indeed exerted by the NK component. By incubating effector cells with low doses of IL-2, we were also able to increase the degree of cytotoxicity against the more resistant blasts. The possibility of expanding NK and NK-like T cells from patients with CLL at diagnosis or after chemotherapy has also been recently demonstrated;[27] the authors proposed that this cell population may be regarded as a potential source for cellular immunotherapy. Most of the studies reported in the literature regarding patients with ALL have failed to generate *in vitro* cytotoxic effectors with lytic activity against autologous blasts. Adult ALL is a very aggressive disease; it is characterized by an overall poor long-term outcome following conventional therapy and most of the patients who reach CR then have early recurrence of disease. Pediatric ALL, which is one of the most frequent neoplastic diseases in childhood, is also an aggressive disease, but has a much more favorable prognosis. So far, the biological characteristics responsible for this different clinical behavior have not been conclusively clarified. New therapeutic approaches, particularly for the management of early disease recurrence or CR consolidation, are warranted.

In this study, we aimed to assess whether: (i) NK cells may be expanded from ALL patients in CR; (ii) the signal transduction machinery of these cells is preserved; (iii) NK cells exert lytic activity against autologous blasts; (iv) IL-2, IL-12 and IL-15 may increase the cytotoxic activity of NK cells against autologous blasts in our *in vitro* model; (v) NK activity and capability of recognizing autologous blasts differ between pediatric and adult ALL patients.

Design and Methods

Patients and controls

Twenty-six patients affected by ALL (11 adults and 15 children) who were in CR and had been off therapy for at least 6 months prior to the study were investigated for their capacity to generate effector NK cells. All patients had been previously treated with chemotherapy or autologous transplantation and remission was evaluated by morphological and flow-cytometric analysis. The target cell population consisted of >95% blasts cryopreserved at diagnosis after sedimentation on a Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient. At the time of the study, blasts were thawed and kept overnight in RPMI 1640 medium (Hyclone, Logan, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) and 2mM glutamine (Hyclone) at 37°C in a humidified 5% CO_2 atmosphere. Peripheral blood samples were collected when the patients were in CR. Normal peripheral blood samples were obtained from healthy donors at the University "La Sapienza" blood bank. All patients and donors gave informed consent to the blood collection and biological studies.

Chemical reagents and antibodies

All chemicals and drugs, unless otherwise mentioned, were obtained from SIGMA (Sigma-Aldrich, Milan, Italy). The following mouse monoclonal antibodies (mAb) were used for immunofluorescence and cytofluorimetric analyses: anti-CD3, anti-CD16, anti-CD56, anti-CD14 and anti-CD19 were purchased from Becton Dickinson (San Jose, CA, USA). Mouse mAb specific for ζ (IgG1, sc-1239) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine mAb (IgG2b, 05-321) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). A mouse mAb specific for CD16 (B73.1) was

generously provided by Prof. A. Santoni (Università "La Sapienza", Roma, Italy). Affinity purified F(ab')$_2$ goat anti-mouse Ig (GAM) was purchased from Cappel-ICN (Costa Mesa, CA, USA). IL-2 was purchased from Proleukin (Chiron, Amsterdam, The Netherlands), while IL-12 and IL-15 were obtained from R&D systems Inc. (Minneapolis, MN, USA).

Cell surface staining and cytofluorimetric analysis

Two-color immunofluorescence was performed, before and after the expansion period, as follows: cell aliquots (5×10^5) were incubated with the fluorescein isothiocyanate (FITC)- or phycoerythin (PE)-conjugated antibody for 30 min at 4°C. Isotype-specific FITC- or PE-conjugated immunoglobulins or the secondary antibody were used as negative controls in all experiments. Stained cells were analyzed on a FACScan cytofluorimeter (Becton Dickinson).

Cell separation, culture conditions and expansion of effector cell populations

Peripheral blood mononuclear cells from patients and normal donors were obtained by sedimentation on a Lymphoprep density gradient (Nycomed Pharma). The cells recovered from the interface were washed twice and resuspended in RPMI 1640 medium (Hyclone) containing 10% heat-inactivated FBS (Hyclone) and glutamine (Hyclone); viability and cell counts were then determined by the trypan blue assay and cytofluorimetric analysis was performed in order to calculate NK cell numbers before the expansion procedure. Cells were then allowed to adhere to plastic for 2 hrs at 37°C in a humidified 5% CO_2 atmosphere in order to remove adherent cells. Polyclonal NK cell cultures were obtained by co-culturing non-adherent PBMC (4×10^5/mL) with irradiated (3000 rad) RPMI 8866 cells (an EBV+ lymphoblastoid B-cell line) (1×10^5/mL) for 10-12 days at 37°C in a humidified 5% CO_2 atmosphere, as previously described.[26] The cell populations used in the experiments were routinely composed of 60-90% $CD56^+CD16^+CD3^-$ cells, as assessed by cell count, immunofluorescence and cytofluorimetric analysis performed at the end of the culture period. During the last 24 hours of the culture period, IL-2 (100 U/mL), IL-12 (10 ng/mL), IL-15 (50 ng/mL), IL-2 (10 U/mL) plus IL-12 (10 ng/mL) or IL-2 (10 U/mL) plus IL-15 (50 ng/mL) were added to the culture medium.

Activation of expanded cells through the CD16 receptor

Expanded cells (5×10^6 cells/300 μL/tube) from adult and pediatric ALL patients were incubated with saturating doses of the anti-CD16 mAb (B73.1, 0.5 μg×10^6 cells) for 30 min at 4°C, then stimulated for different periods of time (0, 1, 5, 20 min) with soluble GAM (1.5 μg/10^6 cells). Cells were then collected and processed for analysis of ζ chain expression and phosphorylated proteins.

Cell lysis, gel electrophoresis and Western blotting

After the 10 to 12-day culture period, cells were washed twice with RPMI 1640 and lysed at 50×10^6/mL for 30 min at 4°C in PBS containing 1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA (pH 8), 100 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μM Na_3VO_4, and 50 mM NaF; equal amounts of lysates for each sample were cleared of debris by centrifugation at 14,000×g for 15 min, resuspended in sample buffer, resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 14% gradient polyacrylamide minigels (Novex Experimental Technologies, San Diego, CA, USA) and transferred to Immobilon-P nitrocellulose membranes (Millipore, Bedford MA, USA). After blocking non-specific reactivity, filters were probed with specific antibodies diluted in TBS-T (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween-20). After extensive washing, immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham, Aylesbury, UK).

Cytotoxic assay

A standard ^{51}Cr release assay was used, as previously described.[26] Target cells (5×10^5) were incubated with 3.7 MBq of ^{51}Cr for 1 hr (K562 and Raji leukemic cell lines) or for 3 hrs (leukemic blast cells of patients) at 37°C and then washed twice with complete medium. Total volumes of 150 μL of complete medium containing 2×10^3 labeled target cells and effectors cells at final effector target (E:T) ratios of 50:1, 25:1, 12.5:1, 6.2:1, 3.1:1, 1.5:1 were placed into V-bottomed microtiter plates. The plates were incubated at 37°C for 4 hrs and then centrifuged at 1200 rpm for 10 min. An aliquot (100 μL) of the supernatant was collected and counted in a γ counter. All experiments were performed in triplicate and the percentage of ^{51}Cr release calculated according to the following formula: (E-S)/(M-S) × 100, where E is the mean cpm release in the presence of effector cells, S is the mean cpm spontaneously released by target cells incubated with medium alone and M is the cpm release of 100 μL of resuspended labeled cells. Results are reported as percentage of cytotoxicity or lytic units per 10^6 cells, defined as the number of effectors required to produce 10% cytotoxicity of 2×10^3 target cells.[1] Only results of experiments in which the value of S/M release was lower than 25% are included. Data were analyzed with t-tests for paired data. The t-tests were used to test differences in the percentage of cytotoxicity against autologous blasts at the E:T ratio of 25:1. Significance is indicated in Table 2.

Results

Expansion capacity

Polyclonal NK cells from ALL patients in remission phase of disease (n=11 adult and n=15 pediatric ALL patients) showed an expansion capacity comparable to that of normal donors (n=10). Non-adherent PBMC from ALL patients in CR cultured in the presence of irradiated RPMI 8866 cells showed an average 5.1-fold (adult) and 4.7-fold (pediatric) increase in the total cell number at day 10, with the $CD56^{+}CD16^{+}CD3^{-}$ NK fraction representing 86-95% (adult) and 62-85% (pediatric) of the total population, the remaining being $CD3^{+}$ cells (Table 1). With regard to the NK population, an average 45-fold (adult) and 35-fold (pediatric) increase in cell number was observed. These results are comparable with those reported in the literature regarding normal donors[28] and with the results obtained with our normal control samples. The increase in cell number was observed starting from day 6, with the maximum proliferation of NK cells taking place between day 6 and 10. No feeder cells, B cells or monocytes were present at the end of the culture period.

Stimulation of expanded NK cells through the CD16 receptor

At the end of the culture period, cells from 2 adult and 2 pediatric ALL patients were stimulated via the CD16 receptor for different periods of time; total lysates were resolved by SDS-PAGE 14% gradient polyacrylamide gel and transferred to nitrocellulose membranes. Filters were then immunoblotted with specific antibodies against the ζ chain associated with the CD16 receptor and tyrosine phosphorylated protein. Immunoreactivity showed, in both adult and pediatric patients, normal events of activation, with maximum phoshorylation of the ζ chain reached at 1-5 min (Figure 1), indicating that the signal transduction apparatus of effector cells expanded from adult and pediatric ALL patients in CR is preserved and comparable to that of normal donors.

Cytotoxic activity of expanded cells against cell lines and autologous ALL blasts, before and after stimulation with IL-2, IL-12, and IL-15

We then tested the cytotoxic potential of expanded cells from adult and pediatric ALL patients in a standard ^{51}Cr release assay against the target leukemic cell lines K562 and Raji, as well as against autologous ALL blasts cryopreserved at diagnosis. Cytotoxic effectors generated from adult ALL patients showed a lytic activity against the different cell lines that was comparable to the lytic activity of pediatric ALL patients and of normal donors, indicating that the cytotoxic potential of the expanded population of effector cells from

Table 1. Expansion of effector cells and phenotypic analysis.

Source of cells	Expansion of effector cells mean (range) Fold increase
Adult ALL patients (n=11)	5.1 (3.3-8.3)
Pediatric ALL patients (n=15)	4.7 (2.2-8.1)
Normal donors (n=10)	5 (3.5-7)

Source of cells	Phenotype of expanded cells: T cells mean (range) %	NK cells mean (range) %
Adult ALL patients (n=11)	6.3 (3-9)	90.6 (86-95)
Pediatric ALL patients (n=15)	15.6 (11-22)	72.8 (62-85)
Normal donors (n=10)	11.2 (5-15)	81.3 (65-97)

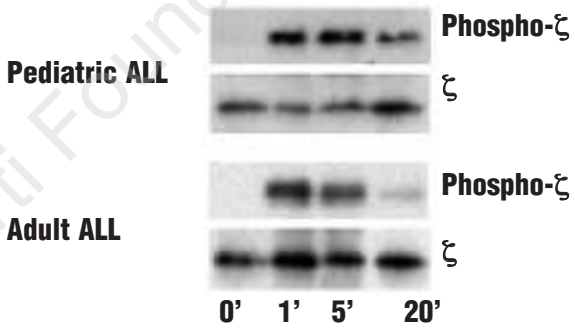

Figure 1. Signal transduction through the CD16 receptor in adult and pediatric ALL patients. Expanded cells from one pediatric and one adult ALL patient were stimulated through the CD16 receptor. Lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) minigels and transferred to nitrocellulose membranes. Filters were probed with monoclonal antibodies against phosphotyrosine and ζ chain. This figure shows representative data from experiments performed in 2 pediatric and 2 adult ALL patients.

ALL patients in CR is preserved. As expected, we obtained the maximum degree of lysis against the NK-susceptible K562 target, while the NK-resistant Raji cell line were lysed to a lesser extent (Figure 2). All cytokines utilized were able to substantially increase the cytotoxic capacity of expanded effectors against tumor cell lines. We then investigated the cytotoxic potential of the expanded cell populations from 3 adult and 4 pediatric ALL patients against autologous blasts. Expanded cells from both adult and pediatric patients, without any activating stimulus by exogenous cytokines, showed very low or no cytotoxic potential (Figures 3 and 4, Table 2). Following incubation of the effector cells with different combinations

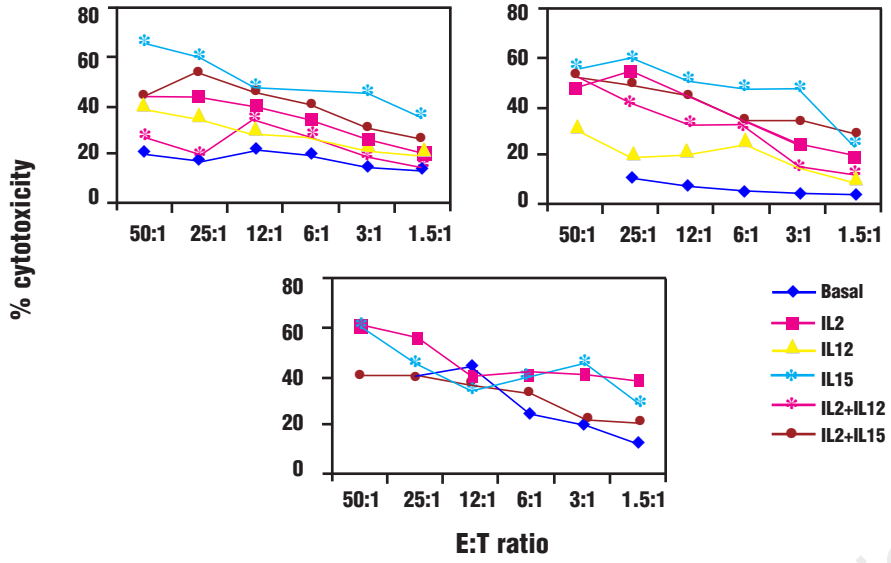

Figure 3. Cytotoxic activity of expanded cells from adult ALL patients against the autologous blast. Expanded cells from 3 adult ALL patients, before and after stimulation with the indicated cytokines, were used as cytotoxic effectors against autologous blasts cryopreserved at diagnosis. Results are expressed as percentage of cytotoxicity.

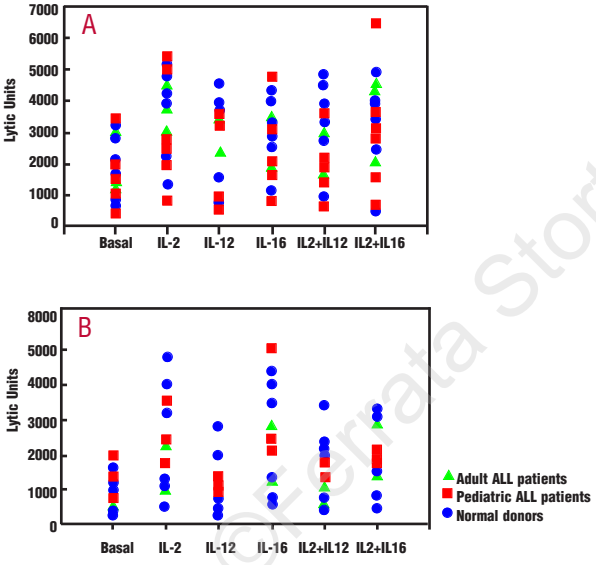

Figure 2. A. Cytotoxic activity of expanded effector cells against the tumor cell line K562. B. Cytotoxic activity of expanded effector cells against the tumor cell line Raji. Expanded cells from normal donors, pediatric and adult ALL patients, before and after stimulation with the indicated cytokines, were used as cytotoxic effectors against the tumor cell line. Each dot represents results from a single experiment. Results are expressed as lytic units.

Table 2. Statistical analysis.

	Mean	*Range*	*Standard Deviation*	*Student's t-test*
Basal (n=7)	12.9	0-30	13.6	
IL-2 (n=7)	38.0	7-69	24.1	*p*=0.009 vs basal *p*=0.06 vs IL-12
IL-12 (n=6)	18.8	0-45	18.0	*p*=0.074 vs basal
IL-15 (n=7)	46.9	12-77	22.1	*p*=0.005 vs basal *p*=0.001 vs IL-12 *p*=0.066 vs IL-2+IL12
IL-2+IL-12 (n=6)	29.3	13-66	20.6	*p*=0.024 vs basal
IL-2+IL-15 (n=7)	43.4	19-80	20.1	*p*=0.007 vs basal *p*=0.000 vs IL-12 *p*=0.013 vs IL-2+IL-12

Differences in the percentage of cytotoxicity at the effector to target ratio of 25:1 against autologous blasts. Significance is indicated.

of activating cytokines, this cytotoxic effect against autologous blasts was substantially increased. In our *in vitro* model, IL-2 and IL-15, alone or in combination, exerted the greatest activating stimulus; in one of the analyzed pediatric cases, after incubating effector cells with IL-2 10 U/mL + IL-15 50 ng/mL, we observed almost 100% cytotoxicity against autologous blasts.

Discussion

Convincing evidence has been provided to support the role of immunological strategies for the management of leukemic patients. The observation that, after related or non-related donor stem cell transplantation, the graft-versus-leukemia response to residual disease is based on immunological mechanisms represented the first real proof of the potential of therapeutic approaches based on immunological recognition;[29,30] the success of therapeutic donor leukocyte infusion in the treatment of relapse definitively confirmed this hypothesis.[31-34]

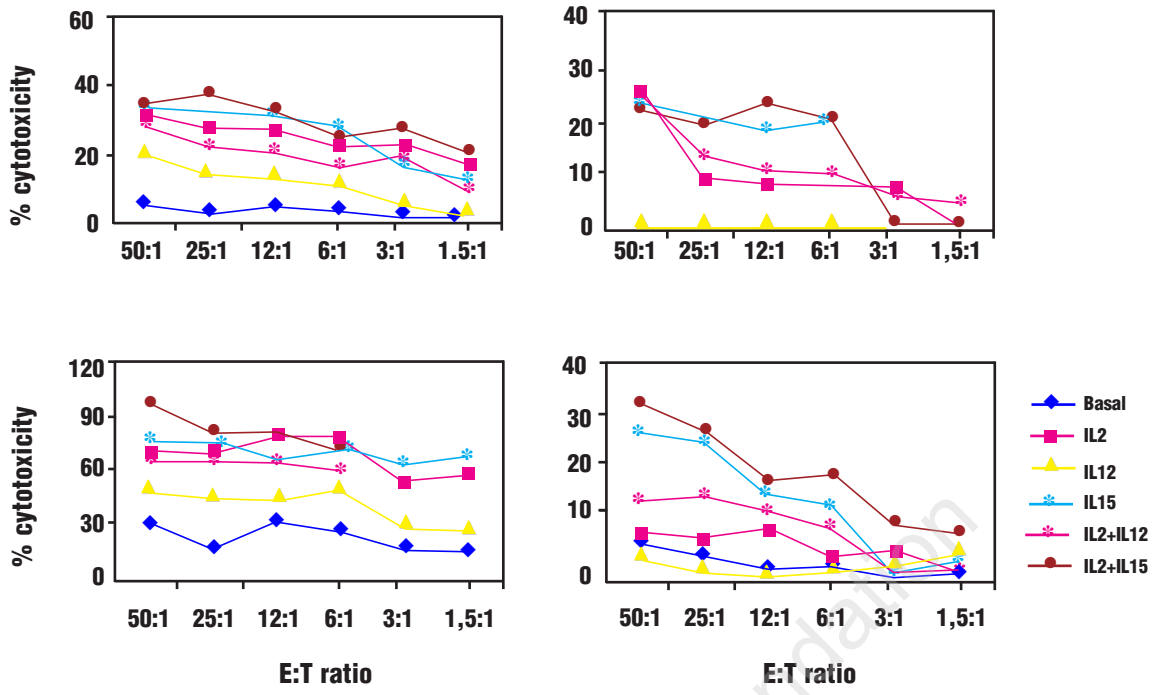

Figure 4. Cytotoxic activity of expanded cells from pediatric ALL patients against the autologous blast. Expanded cells from 4 pediatric ALL patients, before and after stimulation with the indicated cytokines, were used as cytotoxic effectors against autologous blasts cryopreserved at diagnosis. Results are expressed as percentage of cytotoxicity.

Most of these studies were performed in patients affected by myelogenous leukemia. It is well known that lymphoid blasts are more resistant than myeloid cells to immunological recognition and killing by cytotoxic effectors. This is even more evident in the autologous setting; most of the *in vitro* and *in vivo* experimental reports have, in fact, been generated in AML patients. Lowdell and colleagues measured the degree of cell-mediated cytotoxicity in 25 patients with acute leukemia in CR and demonstrated that patients who relapsed had significantly lower cytotoxic activity;[35] interestingly, AML and pediatric ALL patients showed a certain degree of cytotoxic activity, while all adult ALL patients displayed almost no activity. Linn and colleagues recently reported the generation of cytokine-induced killer cells with *in vitro* cytotoxicity against autologous myeloid leukemic blasts, but failed to demonstrate the same activity against lymphoid blast cells.[36] These findings are in agreement with the *in vivo* observations reported in the literature. Consolidative IL-2 therapy has not been found to be useful in preventing relapse in ALL patients after autologous transplantation,[37] while its clinical efficacy has been demonstrated, and confirmed after a follow-up of 10 years, in AML patients.[10,11] The data reported here demonstrate the possibility of expanding cytotoxic effectors, mainly NK cells, from adult and pediatric ALL patients in CR. Under the same culture conditions that enable the expansion of cytotoxic effectors from normal PBMC,[28] a similar 40-fold expansion could be consistently achieved from the peripheral blood of these patients. Expanded cells appear to have an intact signal transduction apparatus, assessed by early events of phosphorylation after triggering the CD16 receptors. The cytotoxic activity against NK-susceptible and NK-resistant tumor cell lines was comparable to that observed from normal donors. We also observed a low basal cytotoxic activity against autologous blasts cryopreserved at diagnosis. In an attempt to increase this level of cytotoxicity, we incubated the NK cells for 24 hours with different combinations of activating cytokines. IL-15 and IL-2 maximally increased the cytotoxic activity against autologous blasts in all the tested cases, both in adult and in pediatric ALL patients, also against the more resistant blasts. It is possible that the modest presence of T cells (3-22%) in the population of expanded effectors may have contributed to the observed cytotoxic activity. We have previously demonstrated, following purification of the NK component, that most of the lytic activity exerted by effector cells expanded from AML patients is indeed exerted by NK cells.[26] The evidence that the immunological compartment of both adult and pediatric patients in CR is evocable, suggests that the different prognoses that characterize these two groups of patients are not related to a different cyto-

toxic potential.

To our knowledge, this is the first report in which such levels of cytotoxicity have been reached against ALL blasts in the autologous setting. We may speculate that stimulation with IL-2 and IL-15 induces the expression of killer activating receptors at the NK cell surface level to an intensity able to prevail over the activity of the matching inhibitory receptors. This hypothesis must of course be examined in future studies aimed at understanding the biological mechanisms responsible for leukemic blast recognition. These results are of particular interest if we consider the high rate of relapse that occurs in adult ALL patients.

While different immunological strategies have been used, with some degree of success, to control minimal residual disease (MRD) in AML patients, including the anti-leukemic activity of NK clones of donor origin in the haplo-identical stem cell transplantation setting,[5] the same cannot be said for ALL patients. The clinical management of these patients has gone through radical changes during the last decade. Subgroups of patients with different prognoses have been identified and different therapeutic protocols (including targeted and biological therapies) have been applied.

In this context, the possibility of monitoring MRD with precise and sensitive innovative techniques represents a key element for selecting the most appropriate therapeutic protocol; it is also possible to evaluate modifications of the residual pathological clone, thus allowing ongoing and appropriate adjustment of the therapeutic strategy. All procedures that may be applied to control MRD have therefore generated great interest; the results reported here suggest a new possible immunotherapeutic strategy that may be considered for the management of ALL patients.

These effectors expanded *ex vivo* may be used for vaccination programs aimed at controlling or eradicating MRD in ALL patients in clinical and hematologic CR. PBMC may be collected at the time of remission and either immediately expanded and infused, or cryopreserved to be subsequently expanded in the presence of persistent disease or early relapse. Moreover, it is possible that these innovative immunotherapeutic approaches, which do not require the infusion of cytokines nor the prerequisite of a preceding allograft, will not have important toxicity. IL-2 and/or IL-15 should be added to the culture medium during the last 24 hours of the expansion period, and not directly infused into the patients as required in other therapeutic protocols, thus possibly avoiding the toxicities and side-effects that are associated with *in vivo* cytokine infusions; this needs to be confirmed by *in vivo* studies. Alongside research to unravel the mechanisms responsible for the phenomena reported here, the feasibility of using autologous cytotoxic effector cells expanded *ex vivo* for the management of ALL patients is worthy of being further explored in order to verify the clinical potential of this procedure.

GFT, AG and RF were responsible for study design, analysis and interpretation of the data. GFT and RM were responsible for cell separation, cytofluorimetric analyses, cell cultures and Western blot analyses. AG and RM were responsible for cytotoxic assays. AG performed the statistical analysis. CA and AV were responsible for the care and follow-up of patients and collection of biological samples. AV was in charge of cryopreserved materials, blast cells stored at diagnosis, and choice of patients to be analyzed. RF was responsible for the general supervision of the research group, for obtaining funding and for critically reviewing the manuscript. All authors have approved the version of the article to be published. The authors declare that they have no potential conflicts of interest.

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