

Interleukin-15 enhances natural killer cell cytotoxicity in patients with acute myeloid leukemia by upregulating the activating NK cell receptors

Mirosław J. Szczepanski · Marta Szajnik · Ann Welsh ·
Kenneth A. Foon · Theresa L. Whiteside ·
Michael Boyiadzis

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Abstract Interleukin-15 (IL-15) has a major role in NK-cell homeostasis. Modulation of the relative frequency and expression intensity of the NK-cell receptors by IL-15 may increase NK cell-mediated cytotoxicity in cancer patients. We investigated the receptor repertoire and measured NK-cell activity in newly diagnosed AML patients and evaluated the ex vivo effects of IL-15. The expression of the activating NK cell receptors was significantly decreased in the AML patients compared to that in NK cells of healthy donors. When NK cells obtained from AML patients were cultured with IL-15, expression of the activating receptors was significantly upregulated compared to pre-culture levels. Concomitantly, cytotoxic activity of NK cells against autologous leukemic blasts increased following IL-15 stimulation. This IL-15 induced increase in activity was blocked by neutralizing antibodies specific for the NK cell activating receptors. These pre-clinical data support the future use of IL-15 for NK cell-based therapies for AML patients.

Keywords Acute myeloid leukemia · Natural killer cells · Activating receptors · NCRs · NKG2D · Interleukin -15

Introduction

Natural killer (NK) cells play a critical role in innate immunity through their capacity to lyse malignant cells without prior antigen-specific priming. Human peripheral blood NK cells can be divided into two functional subsets: (a) CD56^{dim}CD16^{bright} which represent ~90% of all NK cells and mediate cytotoxicity and (b) CD56^{bright}CD16⁻, the remaining 10% of NK cells, which regulate immune responses via cytokine production [1].

Natural killer cell-mediated cytotoxicity is controlled through the balance of signals mediated by activating and inhibitory receptors found on each NK cell, which act as a fail-safe system. Thus, the engagement of activating receptors identifies potential targets, while signaling via inhibitory receptors blocks killing of normal autologous cells [2]. Multiple structurally distinct activating NK cell receptors have been characterized [2]. Those linked to NK cell-mediated killing of tumor cells are NKG2D and natural cytotoxicity receptors (NCR). NKG2D, a C-type lectin-like receptor, recognizes two distinct families of ligands, the MHC class I chain-related molecules (MICA and MICB) and the UL-16 binding proteins (ULBP) [3, 4]. These NKG2D ligands are overexpressed in malignant cells, and NK cytotoxicity correlates with expression levels of MICA, MICB and ULBP on tumor cell targets [5–7]. The NCR such as NKp30 and NKp46, which bind to as yet unidentified tumor antigens, are constitutively expressed on both resting and activated NK cells and are involved in NK cell-mediated killing of a variety of cancer cell targets. Inhibitory NK cell receptors include the killer immunoglobulin-like receptors (KIRs) that

M. J. Szczepanski · M. Szajnik · A. Welsh · K. A. Foon ·
T. L. Whiteside · M. Boyiadzis (✉)
Division of Hematology-Oncology, Department of Medicine,
University of Pittsburgh Cancer Institute,
University of Pittsburgh School of Medicine,
5150 Centre Avenue, Suite 572, Pittsburgh, PA 15232, USA
e-mail: boyiadzism@upmc.edu

Present Address:

M. J. Szczepanski
Departments of Clinical Immunology and Otolaryngology,
Poznan University of Medical Sciences,
61-701 Poznan, Poland

T. L. Whiteside
Department of Pathology,
University of Pittsburgh School of Medicine,
Pittsburgh, PA, USA

bind to classical MHC Class Ia ligands (HLA-A, -B or -C) and the CD94–NKG2A C-type lectin receptor heterodimers that recognize leader peptides derived from HLA-A, -B or -C presented by the non-classical class I molecule, HLA-E [1, 8]. Normal autologous cells which express self-MHC class I ligands are not susceptible to NK cell-mediated killing, but cells that have reduced ligand expression due to malignant transformation are a subject to NK-cell attack [9].

Acute myeloid leukemia (AML) is a disease with considerable phenotypic and genotypic heterogeneity, which is characterized by acquisition of somatic mutations in hematopoietic progenitors. These progenitors acquire a proliferative and/or survival advantage and impair hematopoietic differentiation. NK cells are derived from hematopoietic progenitors in the bone marrow. Patients with AML have depressed NK cell function and cytokine production [10]. Further, NK cell activity correlates positively with relapse-free survival [11]. The inability of NK cells to kill autologous leukemic blasts has also been linked to low expression of NCR, and the NCR^{dull} phenotype has been associated with decreased survival in AML patients [12].

As previously demonstrated, IL-2 and IL-12 play an important role in NK cell biology [13–15]. However, in the last few years IL-15 has been shown to be the major NK cell homeostatic cytokine. NK cell differentiation is cytokine dependent, and interleukin-15 (IL-15) plays a major role in NK-cell differentiation, their expansion in the periphery and survival [16, 17]. Mice lacking expression of either IL-15 or the IL-15R α chain fail to develop NK cells, and NK cells transferred into IL-15 $-/-$ hosts fail to survive [18]. In man, IL-15 induces NK cell differentiation in stroma-free cultures from CD34⁺ progenitor cells and expands mature NK cells in culture. We have demonstrated that mature human circulating NK cells treated with IL-15 up-regulate the expression of their receptors, and this up-regulation is associated with a concomitant increase of the NK cell activity [19].

Based on the significant role of IL-15 in NK cell regulation, we hypothesize that IL-15 may enhance anti-leukemic effects of autologous NK cells in patients with AML either through increasing the NK cell frequency or by alternating the intensity of NK-cell receptor expression. Here, we evaluate the *ex vivo* effects of IL-15 on the NK-cell subsets, their receptor repertoire and NK cell activity in the peripheral blood of patients with newly diagnosed AML prior to any treatment and in normal donors.

Materials and methods

AML patients and healthy volunteers

Blood samples were obtained from newly diagnosed AML patients prior to any treatment ($n = 17$) and from

age-matched healthy volunteers ($n = 10$). All subjects signed an informed consent approved by the Institutional Review Board at the University of Pittsburgh. The collected blood (20–50 mL) was drawn into heparinized tubes. The samples were hand-carried to the laboratory and immediately processed using Ficoll–Hypaque gradients. PBMC were recovered, washed in AIM-V medium (Invitrogen, Carlsbad, CA, USA) counted in a trypan blue dye and immediately used for experiments.

Immunophenotyping

The two NK subsets (CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻) and their receptor expression were evaluated in PBMC obtained from AML patients and normal controls (NC). The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: CD16-FITC, NKG2D-PE, CD25-PE all from BD Pharmingen, San Diego, CA; CD3-ECD, CD56-PC5, CD94-PE, NKp30-PE, NKp44-PE, NKp46-PE, CD158a-PE, CD158b-PE, CD158e1/e2-PE, CD158i-PE, CD122-PE all from Beckman Coulter, Miami, FL; and NKG2A-PE and NKG2C-PE both from R&D System, Minneapolis, MN. Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions. Isotype controls were included in all experiments. Flow cytometry was performed using a Beckman Coulter flow cytometer equipped with the Expo32 software.

Cell culture and IL-15 stimulation

Natural killer cells were isolated from PBMC of healthy donors and from patients with AML using magnetic beads (AutoMACS, Miltenyi Biotec, Auburn, CA, USA) or flow cytometry sorting. NK cells were cultured in the CM—RPMI-1640 complete medium (Invitrogen-Lifetech) supplemented with 10% fetal calf serum (FCS), L-glutamine, sodium pyruvate, non-essential amino acids and penicillin/streptomycin). Cells cultured in the presence of rhIL-15 (Peprotech, Rocky Hill, NJ, USA) at concentrations ranging from 10 to 200 ng/mL were harvested after 24 and 72 h, and the NK receptor repertoire were assessed by multiparameter flow cytometry.

NK cell cytotoxicity

Natural killer cell activity was measured in 4 h ⁵¹Cr release assays using K562 cells as well as autologous myeloid blasts as targets as previously described [20]. Briefly, target cells were labeled with 100 μ Ci of ⁵¹Cr for 1 h at 37°C at 5% CO₂, washed twice in a complete medium (CM), resuspended in CM, and counted. Co-cultures of PBMC and target cells established in triplicate at the effector

to target (*E:T*) cell ratios of 6, 3, 1.5, and 0.75–1 were co-incubated for 4 h at 37°C. Controls included targets incubated in medium alone for spontaneous release and targets in 5% (v/v) Triton X-100 in PBS for maximum release. Radioactivity was measured by Wallac Wizard 1470 Automatic Gamma Counter. The percentage of cytotoxic activity was calculated using the following formula: % specific lysis = (sample cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm) × 100%. Lytic units (LU) were calculated as the number of effector cells needed to lyse 20% of 5×10^3 target cells, and expressed as LU/ 10^7 cells [17]. For the blocking assays neutralizing Abs to NKG2D, NKG2C, NKp46, NKp30 were pre-titered at (0.01–100 µg/mL) to determine the optimal concentration and added to NK cell cultures for 1 h prior to cytotoxicity assays.

Interleukin-15 levels

The IL-15 QuantiGlo ELISA (R&D Systems, Minneapolis, MN, USA) was used to measure the patient's levels of plasma IL-15 prior to any treatment, 14 days after the initiation of induction chemotherapy and in the plasma of patients who achieved complete remission (CR). The sensitivity of the assay was 0.03 pg/mL of IL-15. The ELISA assay was performed in triplicate wells for each patient sample.

Statistical analysis

Data were summarized by descriptive statistics (mean and standard error (SE) for continued variables; frequency and percentage for categorical variables). Statistical analyses were performed using the paired and unpaired two-tailed Student *t* tests. The value of $P < 0.05$ was considered significant.

Results

NK subsets in AML patients

Natural killer cells were evaluated in AML patients at the time of diagnosis and prior to any treatment. The results were compared to those obtained for NK cells of normal controls (NC). The percentage of circulating NK cells was lower ($P < 0.0001$) in the AML patients ($6\% \pm 0.7$, range 1–17%) compared to the NK cells of NC ($12\% \pm 1$, range 9–17%). Relative to NC, the proportion of the $CD56^{\text{bright}}CD16^-$ NK cells in the AML patients was significantly higher, and the frequency of $CD56^{\text{dim}}CD16^+$ cells was significantly lower (Fig. 1).

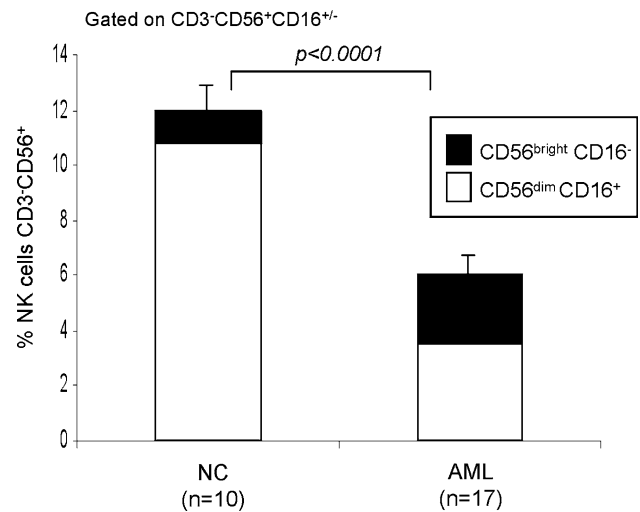


Fig. 1 Percentages of $CD56^{\text{bright}}CD16^-$ and $CD56^{\text{dim}}CD16^+$ in the peripheral blood of AML patients and NC. The data are mean percentages \pm SE

Activating receptor expression in NK cells of AML patients

The frequency of NK cells expressing the activating NCR, NKp30, *NKp44* and NKp46, and the C-type lectin receptors, NKG2D and NKG2C, was significantly decreased in the AML patients compared to the NK cells of NC (Table 1). In addition, the mean fluorescence intensity (MFI) of the activating receptors was significantly lower in AML patients compared to NC (data not shown). We then determined percentages of cells expressing activating NK receptors within the $CD56^{\text{bright}}CD16^-$ and $CD56^{\text{dim}}CD16^+$ subsets of NK cells in the peripheral circulation of AML patients. Both NK cell subsets in these patients contained significantly fewer cells expressing activating receptors compared to the same NK cell subsets in NC (Fig. 2). No

Table 1 Frequency of NK cells expressing activating NK cell receptors in patients with acute myelogenous leukemia and in normal controls

NK cell activating receptors	% Positive		<i>P</i> value
	NK cells in NC (<i>n</i> = 10)	NK cells in AML pts at diagnosis (<i>n</i> = 17)	
NKp30	51	24	0.0001
NKp44	10	4	0.0001
NKp46	73	32	0.0001
NKG2C	28	17	0.03
NKG2D	83	43	0.0001

Percentages of NK cells gated as $CD3^+CD56^+$ cells expressing activating receptors were determined by flow cytometry as described in “Materials and methods”. NC Normal controls

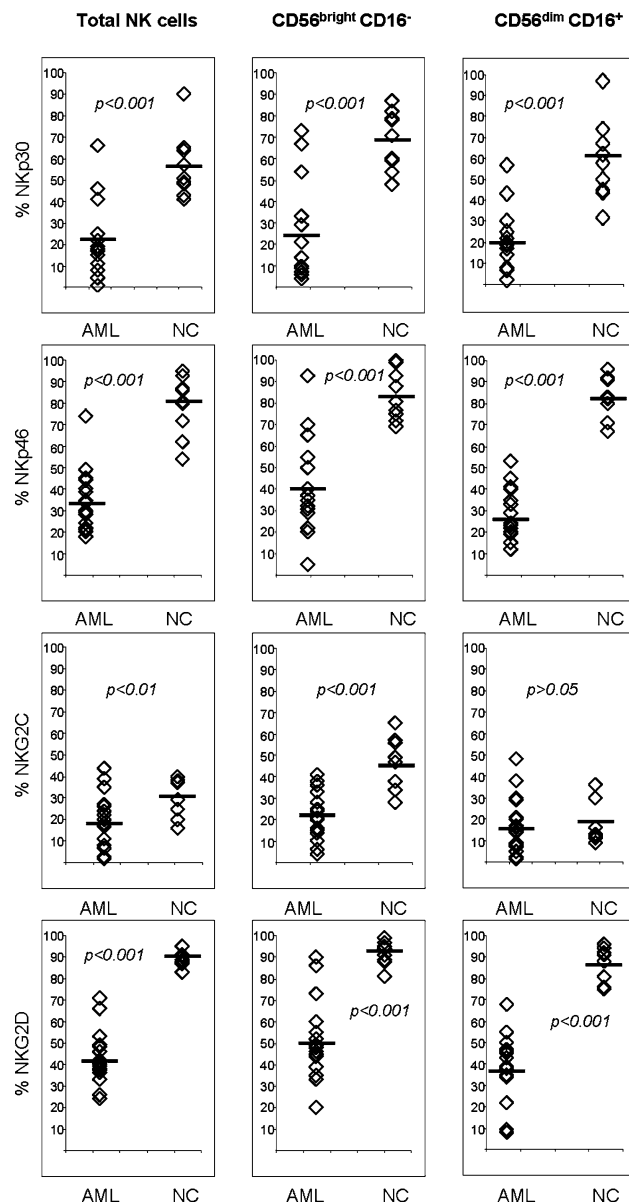


Fig. 2 Percentages of NK cells and NK cell subsets expressing activating receptors in AML patients and NC. Horizontal bars represent the mean values

significant differences in the frequency and MFI of NK cell inhibitory receptors were observed in these subsets between AML patients and NC.

IL-15 increases the expression of activating NK cell receptors in AML patients

To assess the effects of IL-15 on NK cell receptor expression, sorted NK cells were cultured in the presence of IL-15. We observed an up-regulation of the NK cell activating receptors, including an increase in the percentage of cells

Table 2 Effects of IL-15 on the expression of activating receptors in patients with acute myelogenous leukemia

NK cell activating receptors	% Positive		P value
	NK cells in AML pts at diagnosis	NK cells in AML pts after IL-15 stimulation	
NKp30	24	84	0.001
NKp46	32	83	0.001
NKG2C	17	58	0.005
NKG2D	43	87	0.01

Percentages of NK cells gated as CD3⁻CD56⁺ cells expressing activating receptors were determined by flow cytometry as described in “Materials and methods”

expressing the receptors (Table 2) and the MFI for each receptor (Fig. 3). These changes were evident as early as 24 h after the start of culture, reaching a maximum level of expression within 72 h.

IL-15 increases cytotoxicity in cultured NK cells obtained from AML patients

Natural killer cell cytotoxicity was significantly lower in AML patients than in NC (1,102 vs. 1,646 LU, $P < 0.001$). When NK cells sorted from AML patients were cultured with IL-15, NK cell cytotoxicity against K562 targets was increased ($P < 0.05$) (Fig. 4a). When sorted autologous AML blasts were used as targets in cytotoxicity assays, a significant increase in NK cell cytotoxicity against autologous leukemic blasts ($P < 0.05$) was observed (Fig. 4b), which was concomitant with the upregulation of the activating receptors following 3 days of IL-15 stimulation. To assess the contribution of activating receptors on NK cells to the observed increases in NK cell cytotoxicity in response to culture in IL-15, we used neutralizing antibodies specific for the activating receptors in cytotoxicity assays. The addition of the neutralizing antibodies specific for any of the receptors or the simultaneous addition of all four antibodies significantly reduced the level of cytotoxicity (Fig. 4a, b).

NK recovery following induction chemotherapy inversely correlates with plasma IL-15 levels

To further assess the role of IL-15 regulating NK cell behavior in AML patients, we measured plasma IL-15 levels at diagnosis, after induction chemotherapy and when patients achieved a complete remission (CR). At diagnosis, the mean IL-15 level in the patient’s plasma was 1.2 pg/mL (range 0.03–2.29 pg/mL), and it increased

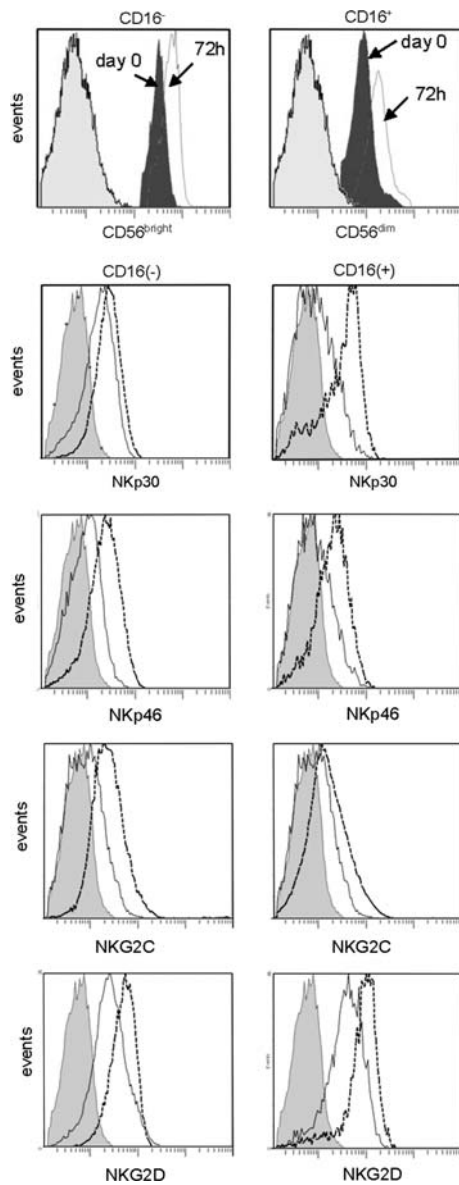


Fig. 3 Up-regulation of expression of NK cell activating receptors by IL-15 in the NK cell subsets in AML patients. Flow cytometry histograms illustrate the levels of expression of NCRs, NKG2D and NKG2C in both NK subsets after culture with IL-15. *Gray-filled histograms* indicate isotype control, *black lines* indicate day 0 values and *dotted lines* indicate expression levels after 72 h culture with IL-15. The representative data were obtained with NK cells of one AML patient

to 5.2 pg/mL (range 0.06–13.4 pg/mL; $P < 0.002$) after induction chemotherapy was completed when the NK cell numbers were reduced to 0 cells/microliter. Subsequently, the level of IL-15 in the plasma decreased to pre-treatment levels when NK cells reconstituted (mean 1.6 pg/mL, range 0.4–2.3 pg/mL) in the AML patients who achieved CR (Fig. 5).

Discussion

In this study, we examined NK cells and their receptor repertoire in patients with AML prior to any treatment. We observed that the $CD56^{\text{bright}}$ NK subset was disproportionately increased in the circulation of newly diagnosed AML patients relative to the $CD56^{\text{dim}}$ subset. It is believed that the progression of $CD56^{\text{bright}}$ to $CD56^{\text{dim}}$ represents the continuum of their normal development and differentiation [1, 21]. The higher proportion of $CD56^{\text{bright}}$ observed in AML patients relative to NC could be due to the increased production of NK cells in response to a rapid replacement of the bone marrow by leukemic blasts or to changes in the cytokine milieu which accompany tumor progression and drive the generation of $CD56^{\text{bright}}$ NK cells.

Activated NK cells have long been known to be effective in killing a broad range of tumor targets. However, NK cell activity in the peripheral blood of untreated acute leukemia patients has been shown to be significantly decreased in this and previous studies [10, 12]. This decrease in NK cell-activity appears to be related to: (a) a lower frequency of NK cells in the peripheral blood of AML patients; (b) up-regulation in expression of MHC class I molecules in leukemia blasts; and (c) decreased expression levels of the ULBP and NCR ligands on the AML blasts [22], possibly as a consequence of the maturation arrest during differentiation of the myelomonocytic cell lineages. Such an arrest could result in an increased resistance of AML targets to NK cell-mediated lysis, and it could also impair the clearance of AML blasts by NK cells bearing the activating NKG2D and NCR receptors. The decreased expression of NK cell activating receptors in AML patients reported here provides yet another explanation for reduced NK cell activity in AML patients. Although the molecular mechanisms responsible for the reduced receptor expression remains elusive, elevated serum levels of soluble NK cell receptor ligands shed by tumor cells have been associated with down-regulation of the NK cell receptors and might contribute to the decreased levels of NK cell activity [23].

Alloreactive NK cells have beneficial anti-tumor effects in haploidentical hematopoietic stem cell transplantation of patients with AML. The enhanced anti-tumor effect of NK cells is mainly due to the incompatibility between the recipient's HLA class I antigens and the KIR repertoire expressed by the donor NK cells [24]. Another consideration for exploiting NK cell anti-tumor effects could be the preferential up-regulation of the NK cell activating receptors. We demonstrated that NK cells from AML patients up-regulate the receptor expression in the presence of exogenous IL-15, with a concomitant increase in receptor-dependent cytotoxicity against NK cell-sensitive tumor targets (K562) and, more importantly, against autologous AML blasts, suggesting that the use of IL-15 can

Fig. 4 Increased NK cell cytotoxicity after IL-15 stimulation of NK cells sorted from the peripheral blood of AML patients. **a** NK cytotoxicity was tested using K562 targets after NK cell culture with IL-15 ± neutralizing Abs to activating receptors. **b** NK cytotoxicity was tested using autologous AML blasts after NK cell culture with IL-15 ± neutralizing Abs to activating receptors at concentrations ranging from 0.1 to 10 µg/mL. The data are mean LU ± SE from 7 experiments (asterisks indicate significant differences)

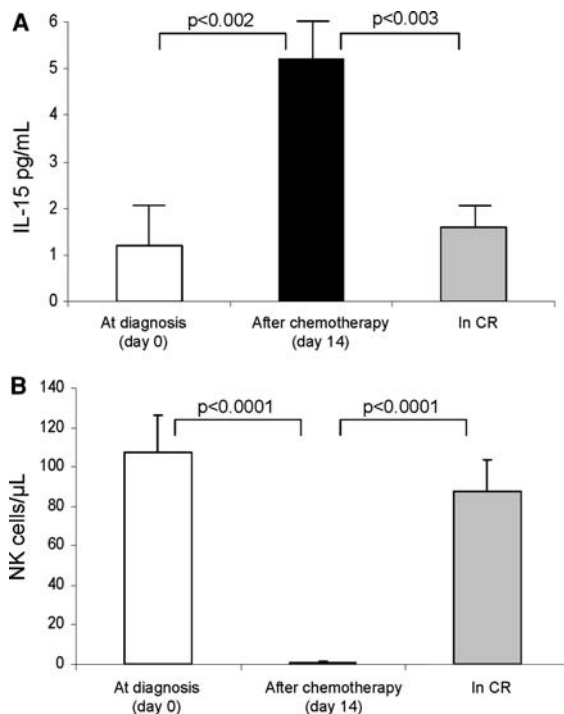
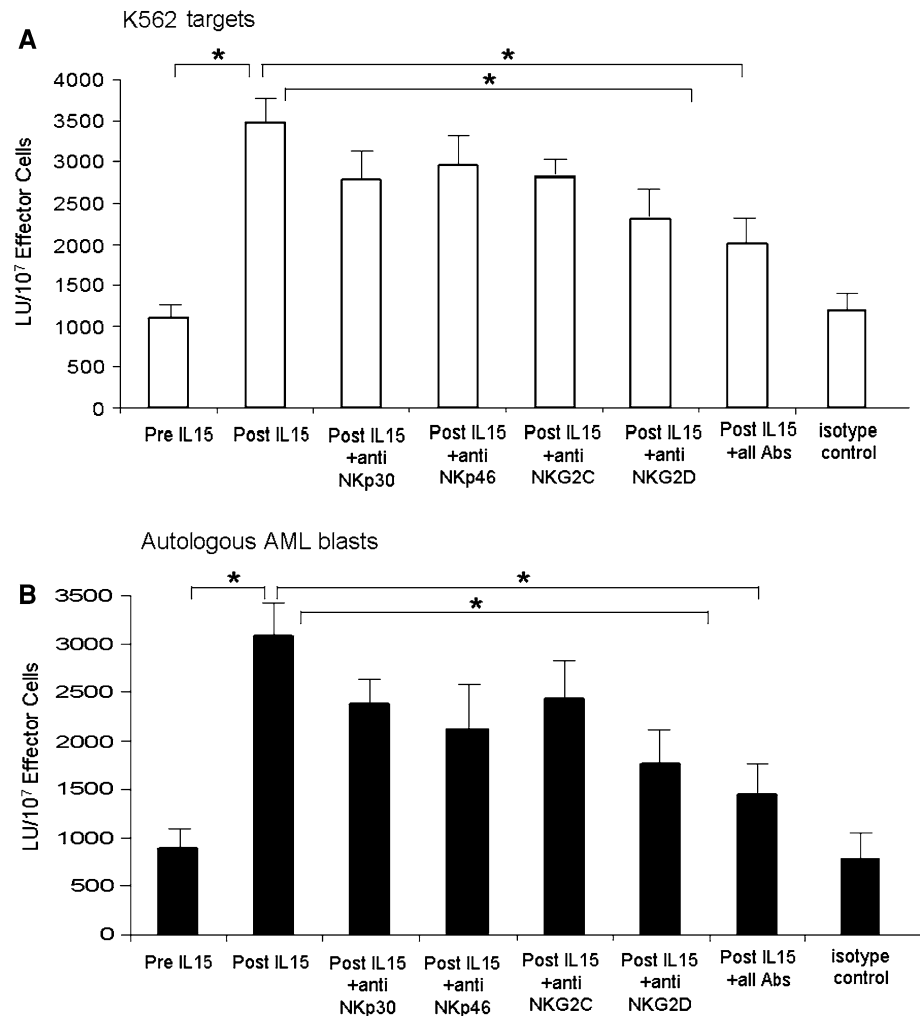


Fig. 5 IL-15 levels in the plasma and NK cell absolute numbers in the AML patients. The levels of IL-15 increased following induction chemotherapy and subsequently declined when NK cells recovered in patients who achieved CR. The data are mean values ± SE

overcome net negative effects of the inhibitory NK cell receptors. These observations are consistent with the concept that IL-15 plays an important role in NK cell anti-tumor activity.

Plasma IL-15 levels have been demonstrated to increase significantly following cytoreductive therapy [25]. We have recently demonstrated that the NK levels in the peri-transplant period are inversely proportional to the dramatic rise and fall in plasma levels of IL-15 [19]. Similarly, in the current study, we found elevated IL-15 levels after induction chemotherapy that decreased following NK cell recovery in those patients who achieved CR. The elevated plasma IL-15 levels could be related to the induction regimen-induced depletion of lymphoid populations that normally consume circulating IL-15 or could be secondary to inflammatory stimulation induced by chemotherapy.

The results of this study demonstrate that NK cells of AML patients acquire high levels of cytotoxic activity against autologous AML blasts upon *ex vivo* stimulation with IL-15. These data support the use of IL-15 in the design of future immunotherapeutic strategies for these patients and for the generation of therapeutic products for adoptive NK cell transfers [26].

Ethical standards

All human studies have been approved by the appropriate ethics committee. Please see “[Materials and methods](#)”, first paragraph.

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Conflict of interest statement All authors agree with the contents of the manuscript and all authors declare that they have no conflict of interest.

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