

Natural killer cells in acute myeloid leukemia patients: from phenotype to transcriptomic analysis

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Abstract Chemotherapies allow complete remission in more than 50 % of patients with acute myeloid leukemia (AML), however, with frequent relapse. This suggests that residual leukemic cells may escape to chemotherapy and immune system. Natural killer (NK) cells from AML patients (AML-NK) have a weaker natural cytotoxicity-activating receptors (NCRs) expression than NK cells from healthy donors (HD-NK). Coding genes for NCR1/NKp46, NCR2/NKp44 and NCR3/NKp30 are located at different loci on two different chromosomes; however, their expression is tightly coordinated. Most NK cells express either high (NCR^{bright}) or low levels (NCR^{dull}) of all three NCRs. This suggests the existence of negative/positive regulation factor(s) common to the three receptors. In order

to find transcription factor(s) or pathway(s) involved in NCRs co-regulation, this study compared the transcriptomic signature of HD-NK and AML-NK cells, before and after in vitro NK cells culture. Microarrays analysis revealed a specific NK cells transcriptomic signature in patients with AML. However, in vitro NK cells expansion erased this signature and up-regulated expression of central molecules of NK functions, such as NCR, NKG2D and also ETS-1, regardless of their origin, i.e., AML-NK vs HD-NK. ETS-1 transcription factor was shown to bind to a specific and common region in the NCRs promoters, thus appearing as a good candidate to explain the coordinated regulation of three NCRs. Such results are encouraging regarding in vitro AML-NK cytotoxicity restoration and provide a new conceptual support for innovative cellular

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therapy based on in vitro NK cells expansion before their reinfusion in AML patients.

Keywords Acute myeloid leukemia · Natural killer cells · Transcriptomic analysis · Natural activating receptor · Gene co-regulation

Abbreviations

CR	Complete remission
NK	Natural killer
AML	Acute myeloid leukemia
TF	Transcription factor
NCR	Natural cytotoxicity receptor
EBV	Epstein barr virus
PBMC	Peripheral blood mononuclear cells
p	<i>p</i> values
FC	Fold change
TFBS	Transcription factor binding sites

Introduction

Acute myeloid leukemia (AML) is characterized by proliferation of immature and abnormal leukocytes, which impede normal hematopoiesis. Although complete remission (CR) is often achieved after chemotherapy, relapse frequently occurs [1–3]. This suggests an escape of residual leukemic cells to chemotherapy and to innate immunity, which plays a pivotal role in the anti-tumoral response [4–6].

Natural killer (NK) lymphocytes are central effectors of innate immunity. Under physiological conditions, normal cells are protected from NK cell-mediated lysis by adequate HLA-class-I-molecules expression. A deficient HLA expression is observed in leukemia cells, which become targets of NK cell-mediated lysis [1, 7, 8]. NK kill tumor cells by recruitment of various activating receptors such as

natural cytotoxicity receptors (NCRs): NCR1/NKp46, NCR2/NKp44 and NCR3/NKp30. Defects of NK functions have been described in many hematological malignancies, including AML [9–14] and >80 % NK cells from healthy donors (HD-NK) express high levels of the three NCRs (NCR^{bright}), while >80 % NK cells from AML patients (AML-NK) express low NCR levels (NCR^{dull}). The anti-tumoral effect of NCRs expression is strengthened by the relatively good prognosis of NCR^{bright} patients compared to NCR^{dull} patients. Recently, two studies highlighted the correlation of NCR^{bright} expression with positive outcomes in AML [15, 16]. Moreover, treatment with IL-2+ Ceplene[®] (histamine dihydrochloride) in AML consolidation, which restores a normal expression (especially of NCR1/NKp46 expression), improves event-free survival in AML [9, 11, 17, 18]. NCR^{dull} phenotype is acquired during leukemia development since a complete (for NCR1/NKp46) or partial (for NCR3/NKp30) reversibility is observed in patients achieving CR after chemotherapy. Reversibility of the NCR^{dull} phenotype after CR, i.e., complete clearance of AML blasts, suggests that leukemia cells might be involved in the down-regulation of NCRs expression. Remarkably, NCRs are encoded by three different loci on two different chromosomes, raising the question of how a coordinated expression can be obtained both in healthy donors (NCR^{bright}) and AML patients (NCR^{dull}).

To solve these questions, analysis was performed in silico to identify putative transcription factor sites of fixation in NCRs promoters in order to find common factor able to explain the coordinated expression of NCRs. Based on recent works [19], this study laid the stress on the transcription factor ETS-1, a potential regulatory element of NCRs expression. In addition, in order to find transcription factors or pathways (which could be implicated in NCRs down-regulation in AML-NK), transcriptomic signature of AML-NK was compared to HD-NK. Transcriptomic data were also compared to the in silico analysis of the NCR promoters.

Patients, materiel and method

Patients and controls

Peripheral blood samples were obtained from 26 patients. Twelve from AML patients treated in our institution (University Hospital La Conception), and 14 from HD, taken in charge in another center (Etablissement Français du Sang Alpes-Méditerranée). The median age of AML patients was 75 years [48–85], and median age of HD was 54 years [19–82]. Patients were classified according to the WHO classification (Table 1). All HD had a NCR^{bright}

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Table 1 AML patients and their clinical characteristics

AML patients	Sex	AML subtypes (WHO)	Bone marrow blasts (%)	Blood blasts (%)	Blood lymphocytes (%)
UPN1	F	Undetermined	28	48	56
UPN2	F	M1	100	36	58
UPN3	F	Undetermined	80	80	51
UPN4	M	M4	20	8	25
UPN5	M	M0	40	19	28
UPN6	F	M5	77	90	9
UPN7	M	Undetermined	30	61	20
UPN8	M	Undetermined	21	3	69
UPN9	M	M6	74	2	12
UPN10	F	M2	57	3	12
UPN11	M	M5	80	47	18
UPN12	F	M4	60	24	28

phenotype except for donor number 940 (NCR^{dull} phenotype). AML patients all had a NCR^{dull} phenotype. In order to facilitate NK cell separation, patients presenting CD45⁺/CD56⁺ leukemic cells phenotype were excluded. The institutional review board of the Assistance Publique des Hôpitaux de Marseille (AP-HM) approved the study, and written informed consent was obtained from each patient.

NK cells expansion

EBV cell line (UMR_S 1040, Montpellier) was used as feeder and was developed to select and specifically activate NK cells by NKG2D, via expression of its ligands: MHC class-I-chain-related protein A (MICA), B (MICB) and UL16-binding protein 1 and 2 (ULBP1 and ULBP2). NK cells had been expanded for 15 days (HD-NK) and for 31 days (AML-NK) from total PBMC cultured at $0.75 \cdot 10^6$ cells/ml with EBV cells feeder at 0.75×10^6 cells/ml in RPMI 1640 medium GlutaMAX (Lifetechnologies, France) with 10 % fetal calf serum (FCS) (from South America, Lifetechnologies, France) in the presence of recombinant human IL-2 (1000 UI/ml) (Sigma, France) at 37 °C in 5 % CO₂.

NK cells sorting

Fresh peripheral blood mononuclear cells (PBMC) were isolated from 6 AML patients and 6 HD, using lymphocyte separation medium density gradient centrifugation (Eurobio Technologies, France). PBMC were labeled with FITC-CD3 and PC5-CD56 (Beckman Coulter, France). CD56-NK cell sorting was achieved with the FACS Aria II (BD Biosciences, France) cell sorter. Purity of obtained NK cell fractions (CD3-/CD56⁺) was >97 %. Sorted cells were stored as dry pellets at -80 °C prior to transcription analysis.

Expanded NK cells were purified by EasySep Human NK Cell Enrichment kit (EasySep, StemCell, France) or by flow cytometry from expansion medium. Purity of NK cell fractions (CD16⁺/CD56⁺) varied from 87 to 98 %.

Immunophenotyping

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), were used to define the NK cells phenotype: CD3 (IgG1), CD56 (IgG1) with following activating receptors: NCR3/NKp30 (IgG1), NCR2/NKp44 (IgG1), NCR1/NKp46 (IgG1), NKG2D (IgG1). All antibodies were purchased from Beckman Coulter (Marseille, France). PBMC, and NK cells were stained with antibodies for 15 min at 4 °C, washed with PBS and analyzed on a FACSCalibur (BD biosciences, Le Pont de Claix, France). Background levels were measured with isotopic controls.

RNA extraction and quantification

Total RNA was extracted using RNeasy Mini kit (Qiagen, France) according to manufacturer instructions. ARN were quantified with NanoDrop spectrophotometer (Thermo scientific, France) and RNA integrity was assessed with Agilent 2100 Bioanalyser (Agilent Technologies).

Transcriptome analysis

Sample amplification, labeling and hybridization followed the Low Input Quick Amp Labeling one-color protocol (version 6.5, May 2010) recommended by Agilent Technologies. Hundred nanograms of each total RNA sample was reverse transcribed into cDNA using oligo dT-T7 promoter primer. Hybridization was performed on whole human genome microarray slides (Agilent©SurePrint G3

V2 8 × 60 K, Agilent Technologies, Santa Clara, CA) containing 24,000 probes with 8200 LincRNA (Long intergenic non-coding RNAs). Hybridized microarray slides were scanned using an Agilent DNA Microarray Scanner and recorded with Agilent Feature Extraction Software (Agilent Technologies). Acquired quantification files were analyzed using the AgiND package (<http://tagc.univmrs.fr/AgiND>), which was also used for quality control and normalization. Quantile methods and background correction were used for data normalization.

Statistical analysis

For each comparison, measurement of differential gene expression was obtained with TIGR Multi-experiment viewer (TMeV) program. Significant Analysis of Microarray (SAM) and Student's *t* test were applied to determine fold changes (FC) and *p* values (P). Data were analyzed using a two-class unpaired response type, which compared control versus AML samples. For each sample comparison, *g*: profiler was used to perform statistical enrichment analysis. The most used source of the functional annotation is Gene Ontology terms [20]. All results were statistically significant with corrected *p* values <0.05 (Bonferroni correction).

Co-culture

After expansion, HD-NK cells were co-cultured with allogenic PBMC from HD or AML leukemic cells (NK/allogenic PBMC or AML blast ratio = 1/5) for five days in RPMI 1640 GlutaMax medium 10 % FCS and IL-2rh (1000U/ml). Malignant NK cell line NK-92 (ATCC, France) was co-cultured 48 h with three different cell lines (1/5 ratio): MOLM-14, KG-1 and Kasumi-1 (ATCC, France), corresponding to AML5, AML6 and AML2, respectively. NK-92 (ATCC) was cultured in α -MEM medium complemented with 2 mM L-glutamine, 1.5 g/l Sodium bicarbonate, 0.2 mM Inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 200–400 U/ml recombinant human IL-2, 12.5 % horse serum and 12.5 % fetal bovine serum.

Reverse transcriptase quantitative PCR (RT-qPCR)

A mix containing optimized concentrations of dNTPs, random primers, oligo (dT) primer and qScript reverse transcriptase was used for cDNA synthesis. Transcripts were quantified by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) on an Stratagene Mx3005P sequence detector (Agilent Technologies) with Applied Biosystems predesigned TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix. The following probes were used (Applied

Biosystems): NCR1/NKp46 (Hs00183118_m1), NCR2/NKp44 (Hs00183113_m1), NCR3/NKp30 (Hs00394809_m1), NKG2D/KLRK1 (Hs00183683_m1), ETS1 (Hs00428293_m1). Housekeeping genes were GAPDH (Hs02758991_g1) and TBP (Hs00427620_m1). TBP showed less variation than GAPDH, that's why this gene was chosen to normalize the results. Results are expressed in relative mean expression \pm SD (standard deviation).

Research of common putative transcription factors binding sites to NCR genes

The MAPPER₂ database (<http://genome.ufl.edu/mapperdb>) is a web-based platform for the analysis of transcription factor binding sites (TFBS) in multiple genomes. The database contains predicted binding sites identified in the promoters of all human, mouse and *Drosophila* genes using 1017 probabilistic models representing over 600 different transcription factors. MAPPER₂ includes three large libraries of TFBS models: TRANSFAC, MAPPER and JASPAR (Riva, 2012). A multiple search was performed on the region –8 Kb upstream transcription starting-site (TSS) of the human NCR1/NKp46, NCR2/NKp44 and NCR3/NKp30 genes. Those sequences were extracted using BioMart (PMID 19144180) in GRCh37/hg19 coordinates from v76 (<http://grch37.ensembl.org/>) annotations (PMID 19906699). The ETS1 motif was retrieved from JASPAR core vertebrates' database (version 5.0_alpha) and shortened to the core motif of 8 bp. The RSAT suite was then used to scan this modified ETS1 motif upstream of NCRs (NCR1/NKp46, NCR2/NKp44 and NCR3/NKp30). This in silico search of ETS1 was performed with oligo-analysis [21] and matrixscan [22] from the RSAT (Regulatory Sequence Analysis Tools) [23] suite with default settings and *p* value <10^{–4} (<http://tagc.univ-mrs.fr/rsa-tools/>).

Chromatin Immunoprecipitation (ChIP)

Approximately 1 × 10⁷ NK-92 cells were suspended in ice-cold PBS. Proteins were cross-linked to the DNA using formaldehyde. After sonication, fragmentation of the DNA was performed to obtain DNA fragment around 200–500 nucleotides with a CovarisTMS2 sonicator. Magnetic Dynabeads protein A/G (Invitrogen) was added to the sample, and the suspension was transferred and divided into two fractions (IP and input). The ETS-1 antibody (C-4) used was a mouse monoclonal IgG₁ antibody (sc-55581) (Santa Cruz biotechnology). The fraction without antibody (input) served as a positive control while mouse non-specific antibodies served as a negative control. The immune complexes were washed, and all washing steps were carried out at 4 °C. Dynabeads protein A/G were

suspended in reverse cross-linking buffer (Invitrogen) and proteinase K and incubated. The DNA was isolated using PCR Purification Kit (Invitrogen) following the manufacturer's instructions. DNA was amplified by quantitative PCR (Syber green 1, Light Cycler[®] 2, Roche applied science) using NCR-specific primers pair. All q-PCR results were replicated and normalized with input. The primers used were:

	Forward	Reverse
NCR1/NKp46	5'-GGAGTTGTGAACGT TCTGATGA-3'	3'-TAGTGCTGAGC AGTGGGGACT-5'
NCR2/NKp44	5'-GGGAAGCAGTCC ATAACTCG-3'	3'-TCCCAGGCCT CTGTAGAAA-5'
NCR3/NKp30	5'-AGGGTCTCTAGG AGGCCAAG-3'	3'-GTGCAGTGA GGCCAGATTC-5'

Results

AML-NK has lower ability to proliferate and weaker NCR expression after in vitro stimulation than HD-NK

Mean NK percentage of NK in PBMC was $2.42\% \pm 2.19$ in AML patients versus $7.6\% \pm 3.18$ in HD ($p < 0.05$) (Data not shown). After 17 days of in vitro expansion, HD-NK cells proliferative index was 312 ± 93 and NK cells percentage in cell culture increased from $7.6\% \pm 3.18$ – $46.8\% \pm 23.4$ ($p < 0.01$). To reach almost the same enrichment values in AML patients ($33.45\% \pm 18.88$ of NK cells and a proliferative index of 79 ± 19), the culture was carried on 31 days. NK cell expansion induced an increase in NCRs and NKG2D expression in all AML-NK (Fig. 1). Comparison of HD-NK cells and AML-NK cells showed that MFI ratios were, respectively, 41 versus 58 % for NCR1/NKp46 ($p < 0.05$), 683 versus 262 % for NCR2/NKp44 ($p < 0.05$), 93 versus 54 % for NCR3/NKp30 ($p < 0.05$), and 240 versus 40 % for NKG2D ($p < 0.05$).

NCRs expression of HD-NK and NK-92 cells are down-regulated in presence of AML blasts

After 5 days of co-culture with AML blasts compared to control group (co-culture with HD-PBMC), MFI for NCR in HD-NK cells decreased of 28 % (12.78 ± 1.70 vs 9.22 ± 0.99 , $p < 0.05$) for NCR1/NKp46, 21 % (15.5 ± 4.65 vs 12.23 ± 3.01 , $p > 0.05$, [NS]) for NCR2/NKp44 and of 20 % (10.19 ± 2.15 vs 8.15 ± 1.76 , $p < 0.05$) for NCR3/NKp30. As control, no significant difference was observed in NKG2D expression (16 % decrease (16.12 ± 3.51 vs 13.53 ± 2.73 , $p > 0.05$, [NS])) (Fig. 2).

Analysis of the NCR messenger RNA level variation following AML blast co-culture by performing RT-qPCR found similar results. Mean expression for NCR1/NKp46 decreased of 42.2 % (57.8 ± 24.5 vs 100 %, $p < 0.05$), NCR2/NKp44 mean expression increased of 4 % (104.3 ± 44.8 , [NS]), NCR3/NKp30 decreased of 43 % (56.9 ± 48 , $p < 0.05$), and NKG2D decreased of 15.7 % (84.2 ± 28.1 , [NS]) (Fig. 3).

Furthermore, after 5 days of co-culture, NCR were also down-regulated in NK-92 co-culture with Molm-14 AML cell line. Compared to control group (NK-92 cultured without AML cell line), NCR MFI in NK-92 cells cultured with Molm-14 decreased of 40.8 % (4.13 ± 0.36 vs 2.44 ± 0.33 , $p < 0.01$) for NCR1/NKp46, of 22.5 % (2.16 ± 0.17 vs 1.67 ± 0.06 , $p < 0.01$) for NCR2/NKp44 and of 29 % (3.36 ± 0.46 vs 2.39 ± 0.20 , $p < 0.05$) for NCR3/NKp30 (Fig. 4).

In silico analysis revealed common putative binding site ETS-1 in NCR promoters

Binding sites of common transcription factors (TF) upstream of the three NCR genes [−8 Kb; TSS] were determined using Mapper2 database (Table 2). Focus was then put on ETS-1 TF, taking into account the involvement of ETS-1 TF in NK cell development and cytotoxicity in mice model. The region [−8 Kb; TSS] of NCR promoters was scanned with the ETS-1 matrix motif from JASPAR database (Fig. 5) using MatrixScan from the RSAT suite. This in silico approach identified several putative binding sites of ETS-1 upstream the three NCRs genes (Data not shown). Among these binding sites, three sites were chosen closest to the TSS and also found in conserved regions between seven different species (rhesus, chimp, mouse, chicken, dog, elephant and zebrafish) defined from GERP elements in 37-way multiple genome alignments (Cite PMID 19906699). These predicted ETS-1 binding sites coordinates were chr19: 54,906,112–54,906,119 (position −76; −69 from TSS with p value = $3.4e-05$) for NCR1/NKp46, chr6: 41,303,270–41,303,277 (position −345; −338 from TSS with p value = $9.1e-04$) for NCR2/NKp44 and chr6: 31,593,068–31,593,076 (position −7917; −7910 from TSS with p value = $9.5e-04$) for NCR3/NKp30 (data not shown).

In NK-92 cells, ETS-1 transcription factor binds in vivo to the in silico predicted region in NCRs promoters

To test the recruitment in vivo of ETS-1 in NK-92 cell line, a pair of primers was designed to perform site-specific chromatin immuno-precipitation (ChIP) assay. After cross-linking and sonication, the chromatin extract was immuno-

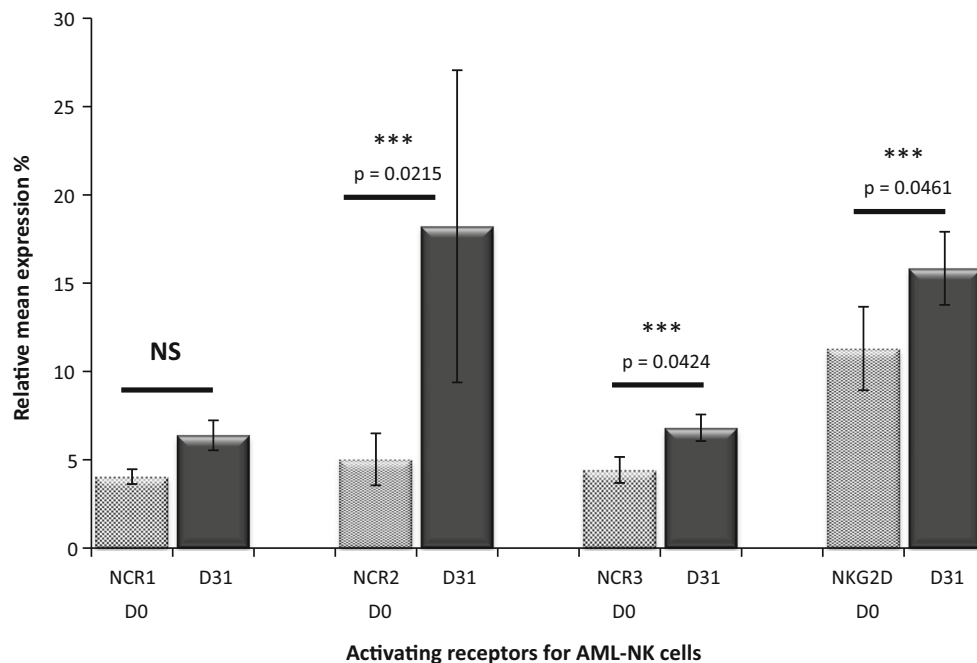


Fig. 1 NCR expression of AML-NK cells after in vitro expansion. Evolution of activating receptors (NCR1, NCR2, NCR3 and NKG2D) expression expressed in mean fluorescence intensity (MFI) compared

to isotypic control, before and after (black bars) NK cells expansion. ***Statistically significant difference ($p < 0.05$). NS no statistically significant difference

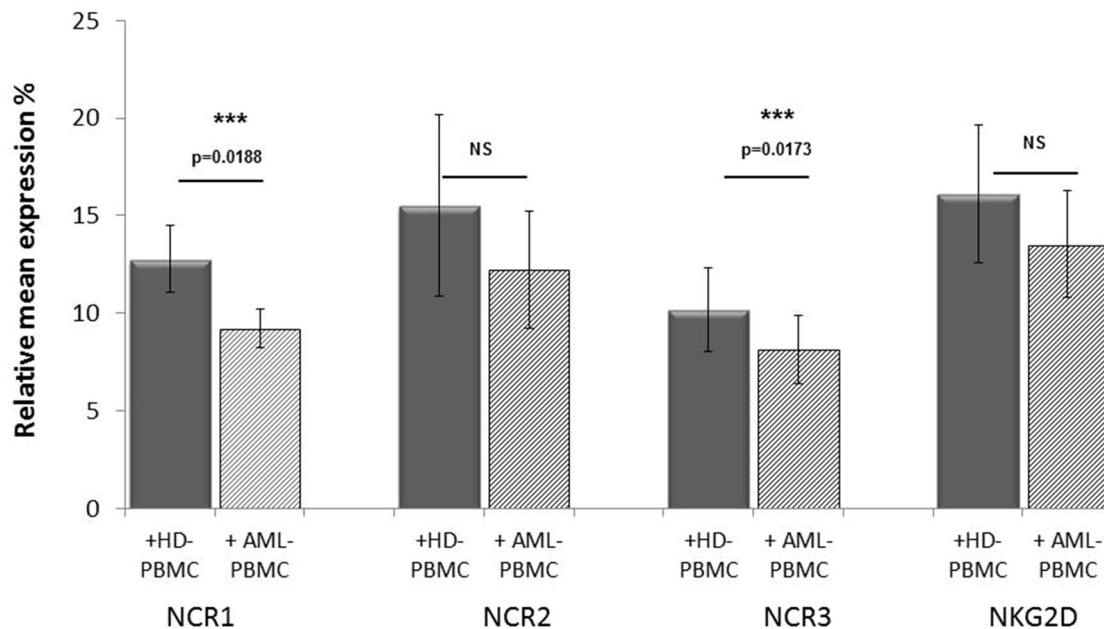


Fig. 2 HD-NK cell activating receptors expression in presence of AML-PBMC. Evolution of NCR1, NCR2, NCR3 and NKG2D expression in mean fluorescence intensity (MFI), background levels were measured using isotypic control, after co-culture of HD-NK

cells with AML-PBMC compared to the co-culture with HD-PBMC (black bars). ***Statistically significant difference ($p < 0.05$). NS no statistically significant difference

precipitated. The three regions of ETS-1 binding sites were amplified by PCR using specific primer pairs. Strong recruitment of ETS-1 on targeted regions upstream the three *NCRs* genes were observed (Fig. 6a, b). These results

are consistent with Electrophoretic Motility Shift Assay (EMSA) that showed a band shift after incubating NCR1, NCR2 and NCR3 sequences containing EST-1 motif with nuclear proteins from NK92 (data not shown).

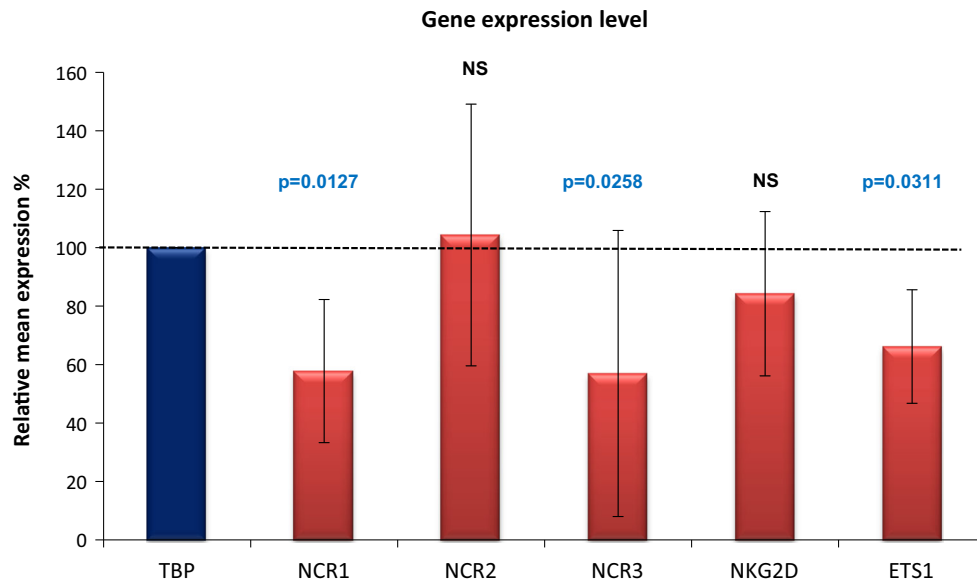


Fig. 3 Gene expression level of activating receptors after co-culture with AML-PBMC. Histograms represent the relative mean expression in percentage of NCR1, NCR2, NCR3 and NKG2D genes when HD-NK cells are co-cultured with PBMC from AML patients, compared

to the same HD-NK cells co-cultured with PBMC from donors (corresponding to 100 %). Results were normalized with TBP housekeeping gene

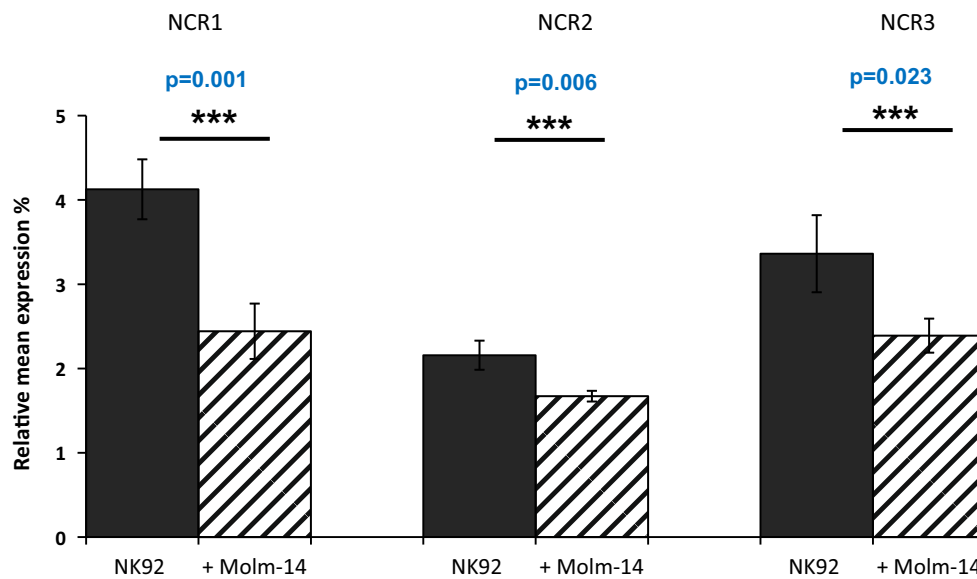


Fig. 4 Activating receptors expression in NK-92 co-cultured with leukemic blasts. Evolution of NCR1, NCR2 and NCR3 expressions, in MFI ratio, after NK-92 co-culture with Molm-14 AML cell line

compared to NK-92 with medium (black bars). ***Statistically significant difference ($p < 0.05$). NS no statistically significant difference

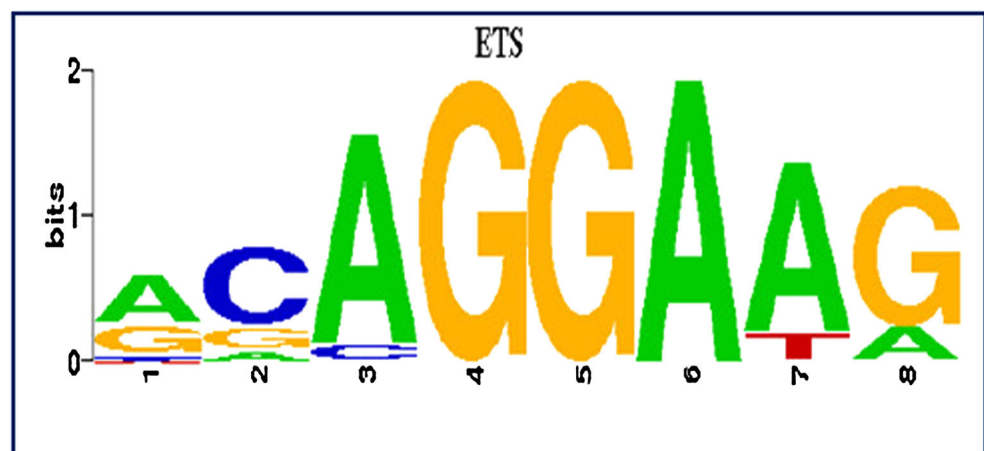
ETS-1 gene expression decreased in presence of AML blasts

In HD-NK cells co-cultured during 5 days with AML blast ETS-1 gene expression level decreased of 34 % (66.1 ± 19.4 vs 100 %; $p < 0.05$) compared to the control group (co-

culture with HD-PBMC, condition of calibration = 100 %) (Fig. 3). ETS-1 transcript level followed the same regulation than NCR1/NKp46 and NCR3/NKp30 mRNA, which, respectively, decreased of 42.2 % (57.8 ± 24.5 vs 100 %, $p < 0.05$) and 43 % (56.9 ± 48 , $p < 0.05$). No evolution (increase or decrease) of NCR2 has been observed.

Table 2 List of common putative binding site of transcription factors upstream NCR genes, in [-8 Kb, TSS] region

Transcription factor	NCR1	NCR2	NCR3
AML1	-171 to 166 -273 to 268	-84 to 79 -265 to 260	-90 to 95
br_Z1	-237 to 225	-26 to 14	-406 to 418
CP2	-179 to 169	-130 to 120	-134 to 144
dl_2	-307 to 298	-105 to 96	-91 to 100
Dof2	-76 to 71 -91 to 86	-334 to 329	-126 to 131
ETS1	-37 to 33 -360 to 356	-257 to 253	-231 to 235
Hand1_Tcfe2a	-243 to 235	-132 to 124	-232 to 240
HMG_1	-405 to 397	-407 to 399	-285 to 293
MZF1_5_13	-122 to 114	-61 to 53	-19 to 27
NFKB1	-307 to 297	-289 to 281 -106 to 96	-91 to 101
PacC	-19 to 14	-439 to 434	-160 to 165
RELA	-338 to 329	-105 to 96 -498 to 489	-91 to 100
RREB1	-71 to 52	-51 to 32 -134 to 115 -477 to 458	-16 to 35 -133 to 152 -212 to 231
Sp1	-16 to 7	-361 to 353	-22 to 30 -184 to 192
Su(H)	-282 to 268 -354 to 340	-93 to 79 -164 to 150	-89 to 103
TP53	-296 to 277 -302 to 283	-459 to 440	-130 to 149
zmf143	-125 to 106 -218 to 199	-224 to 205	-263 to 282

Fig. 5 ETS-1 motif. ETS1 motif was retrieved from JASPAR core vertebrates' database

Microarray analysis of NK after in vitro cell expansion shows a specific transcriptomic signature including ETS-1 up-regulation (Fig. 7)

After conducting a significant analysis of microarray (SAM) with a FDR = 0 % with the four groups (HD-NK ± expansion, AML-NK ± expansion), a major hierarchical clustering was obtained, distinguishing NK cells with and without expansion (Cluster I and II) regardless of the origin of NK (HD-NK vs AML-NK). A specific transcriptomic signature of very few genes allowed distinction of AML-NK from HD-NK cells (see below), although most frequent differences were linked to the presence or absence of NK cells culture. Hierarchical clustering and the two differential clusters (I and II) are showed in the heatmap (Fig. 7a): 5844 probe sets, coding for 3500 genes, were differentially expressed in expanded vs unexpanded NK.

Microarray analysis revealed AML-NK cells specific transcriptomic signature, erased by NK cells expansion. The transcriptomic analysis revealed a specific signature that differentiates AML-NK cells from HD-NK cells. However, cluster (b) separates AML from HD-NK cells only after sorting, not after in vitro expansion. Indeed, NK cells expansion erased the differential transcriptomic signature between NK cells from AML or HD patients. In this cluster, CD86, CLIC4 and CREG1 were related to lymphocytes differentiation and activation. However, remaining genes are not directly involved in AML or NK cells functions or biology according to scientific literature.

A sub-cluster of interest (c) was isolated with the heatmap (a). It was implicated in NK cell-mediated cytotoxicity, with up-regulated expression after culture of NCR1/NKp46, NCR3/NKP30, KLRF1, KLRC1, KLRC3, CD244, PRDM1, CD7, NKG2D and ETS-1, in HD-NK and AML-

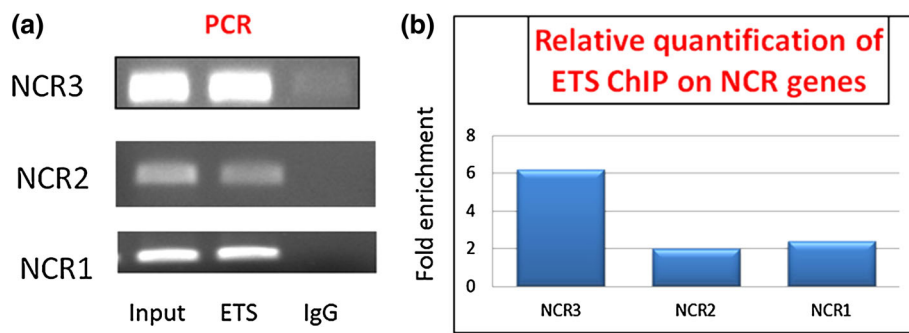


Fig. 6 In vivo recruitment of ETS-1 transcription factor on the human NCR genes promoter. **a** Semi-quantitative PCR results of electrophoresis on an agarose gel. **b** Results of the quantitative

chromatin immunoprecipitation assays performed using specific antibodies for ETS-1 as described in materials and methods

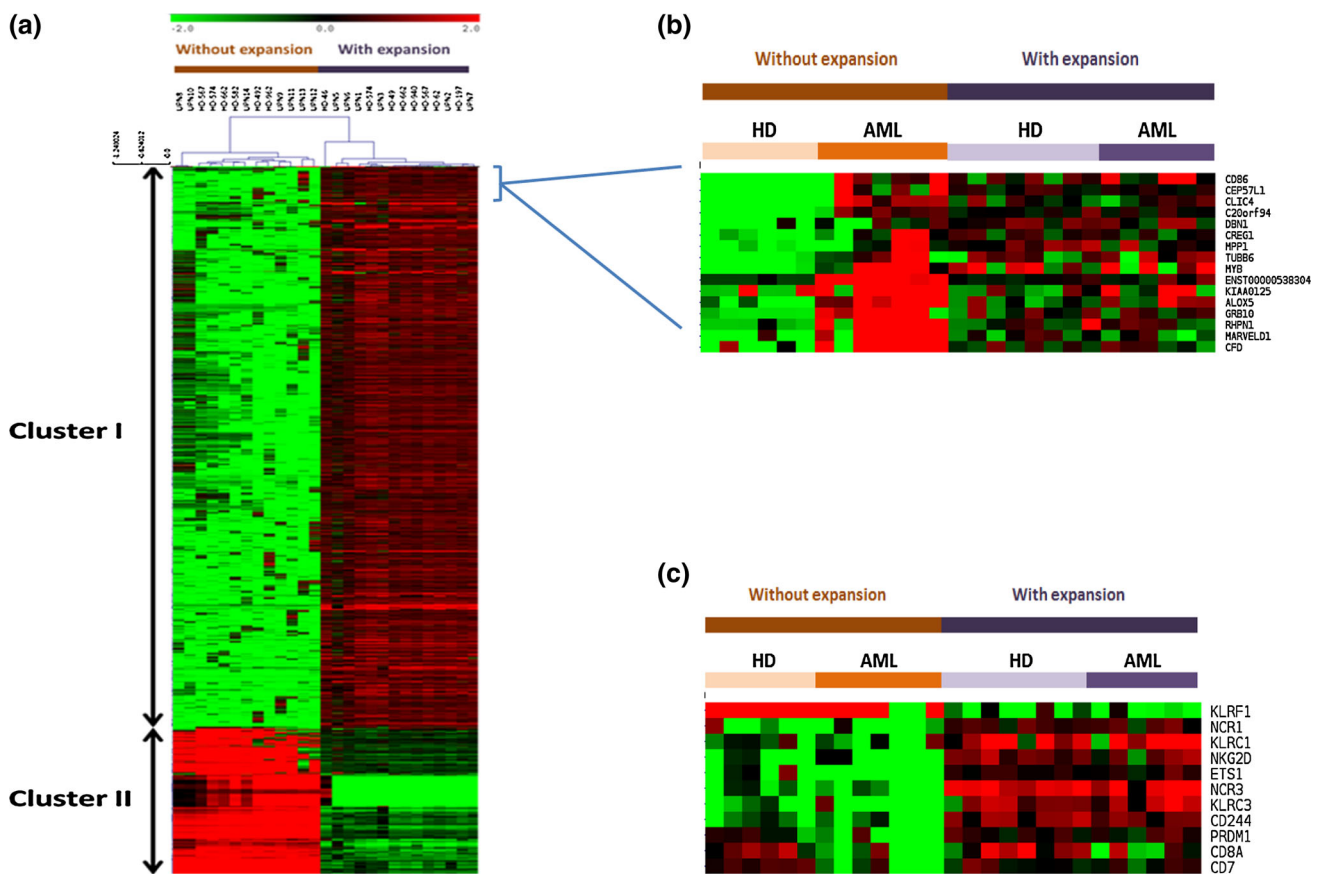


Fig. 7 Heatmap of gene expression changes in AML patients versus donors with and without NK cell expansion. **a** The hierarchical clustering represents overexpressed (red) and underexpressed (green) genes in sorted NK cells from 12 AML patients (UPN) and 14 HD. Normalized signal intensities were treated with the SAM analyze to highlight the differentially expressed genes with a FDR set at 0 %. Major hierarchical clustering distinguishes NK cells with and without expansion (Cluster I and II) regardless of the origin of NK (HD-NK vs AML-NK). **b** A specific transcriptomic signature including very few

genes distinguished AML-NK cells from HD-NK cells. AML-NK cells specific transcriptomic signature. Microarray analysis revealed AML-NK cells specific transcriptomic signature. NK cells expansion erased the differential transcriptomic signature between NK cells from AML patients or HD. **c** Sub-cluster of genes of interest after NK cell expansion. After NK cell expansion genes like NCR1/NKp46, NCR3/NKP30, NKG2D and ETS-1 were significantly up-regulated in AML patients and healthy donors (HD)

NK. Most of the other genes were related to lymphocytes differentiation and activation (CD8A, PRDM1 and FAM113B). Other genes with differential expression

between NK cells with or without expansion failed to give more clues, since their function is still unknown. Expression level of six differentially expressed genes was checked

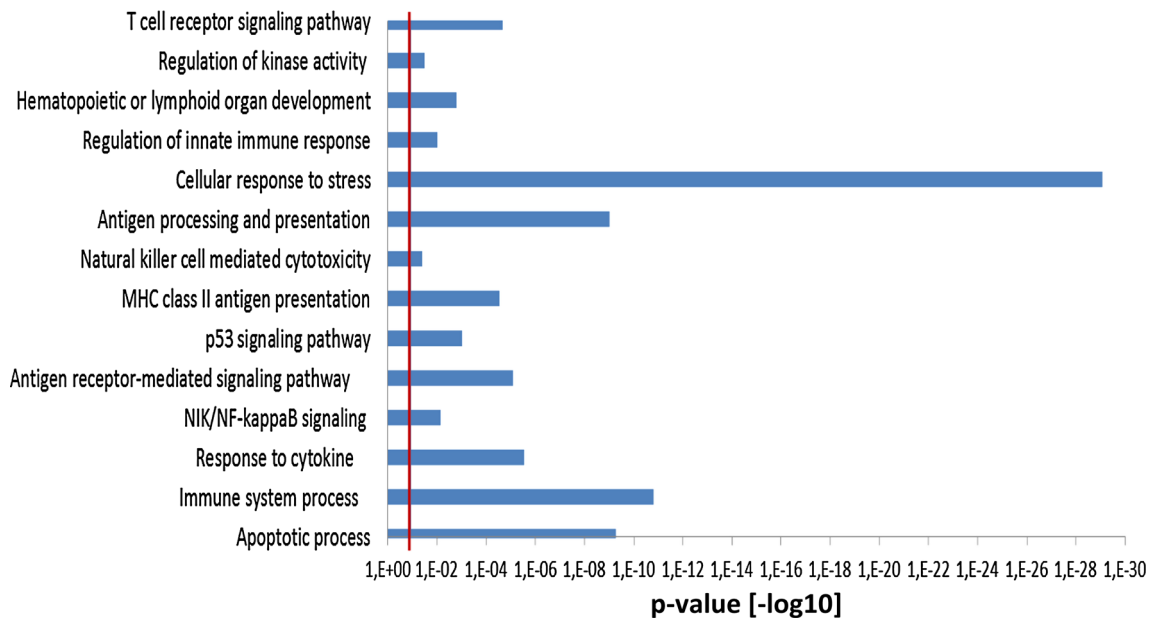


Fig. 8 Functional annotation. Summary of some functional enrichment terms (GO terms) found with g: profiler web server. In ordinate, corrected p values (Bonferroni correction) of each Go term and in abscissa, selected Go terms. All the Go terms are statistically

significant with corrected p values <0.05 . Functional annotations correspond to immune responses, cellular response to stress, and cytokine-related response were the most significant pathways linked to the NK expansion

with RT-qPCR for microarray analysis validation (data not shown).

The functional annotation was performed using g: profiler (Fig. 8). Annotations implied in triggering immune and cellular responses to stress or cytokines-related response were the most significant pathways linked to the NK expansion. All selected pathways were statistically significant with corrected <0.05 p values.

Discussion

Despite stimulation potential of EBV feeder cell line and high concentration of IL-2 used in vitro for NK cell expansion, AML-NK showed slow and low proliferation ability 4 times weaker than HD-NK, even after 30 days of culture. Moreover, only a partial restoration of NK activating receptors was observed in comparison with HD-NK cells. However, this expansion protocol could be suitable for cellular therapy with NK cells activation ex vivo since it enhances NCR1/NKp46 and NCR3/NKp30 expression.

Nonetheless, since the AML-NK results were less positive than with HD-NK, we aimed to better understand the mechanisms underlying this down-regulation by focusing on the regulation of transcription of the three NCRs. Their coordinated expressions led us to search for a common transcription factor and transcription factor ETS-1 seemed particularly interesting since NK cells from ETS-1-

deficient mice have decreased cytotoxicity. At the earliest stages of NK cell development in mice, the ETS-1 factor promotes expression of activating receptors such as NKp46 [19]. However, it is unclear whether these transcription factors play roles in NK cytotoxicity [1, 24–28]. A homologous motif of the ETS-binding site was identified in the mouse promoter that enhanced perforin transcription in cytotoxic cells. This study also identified two DNA-binding proteins that interact with this element, which are thought to be members of the ETS transcription factors family [28, 29]. We have demonstrated that in NK-92 cells, ETS-1 transcription factor (TF) binds in vivo the in silico predicted region in NCR promoters. Moreover, ETS-1 gene expression is decreased in presence of AML blasts and follows the same expression pattern than NCR. However, this experimentation did not allow concluding whether ETS-1 binds directly to the targeted region because many TF cooperate to induce gene transcription in genes regulation. Further studies, especially using RNA interference, should try to refine this hypothesis.

Khaznadar et al. [30] recently described that defective functions of AML-NK are associated with a leukemic blast transcriptional signatures of immune evasion, which questions NK cells defective functions origins, perhaps also linked to a particular specific signature of deficient cytotoxicity.

The transcriptomic comparison of HD-NK and AML-NK, with and without in vitro culture and expansion, allowed two main clusters identification (cluster I and II)

that differentiate primary unexpanded NK cells from expanded/activated NK cells, regardless of their origin (HD versus AML patients). A specific AML-NK signature (Fig. 7b) was detected in unexpanded NK cells and given the genes involved; we do not know to what extent this signature could be specific of defective cytotoxicity leading to innate immunity escape. But interestingly, this specific transcriptomic signature was reversible after NK cells expansion. Remarkably, we noted that, under our specific culture conditions, NK cells expansion up-regulated expression of central molecules of NK functions, i.e., NCR, NKG2D (cytotoxicity) and ETS-1 (a pivotal TF for lymphocytes) in both HD-NK and AML-NK, which was associated with the recovery of a cytotoxic phenotype. Moreover, besides NK cells mediated cytotoxicity, it's more an extensive restoration of the immune functions of NK cells which was observed. Indeed, many pathways implicated in triggering immune responses, cellular response to stress and relating to cytokines response were enhanced after NK cells expansion. Such results are encouraging regarding our ability to restore in vitro AML-NK cytotoxicity in all levels (from genes expression to phenotype) and provides a new conceptual support for innovative cellular therapy based on NK cells expansion in vitro before reinfusion in AML patients.

In conclusion, NK cells from AML patients showed slow and low proliferation ability that was 4 times weaker than HD-NK, even after 30 days of culture. Only a partial restoration of NK activating receptors was observed in comparison with HD-NK cells. Microarrays analysis revealed a NK cells specific transcriptomic signature in patients with acute myeloid leukemia. However, NK cells expansion erased this signature in vitro and up-regulated expression of central molecules of NK functions, such as NCR, NKG2D and ETS-1, suggesting ETS-1 transcription factor may play a major role in NCR co-regulation. ETS-1 binds in vivo to an in silico predicted region in NCR promoters and its expression decreased in presence of AML blasts, following the same expression as NCR.

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Compliance with ethical standards

Conflict of interest All authors declare no financial conflict of interest.

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