Calreticulin exposure on malignant blasts correlates with improved natural killer cell-mediated cytotoxicity in acute myeloid leukemia patients

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Supplementary data

Supplemental Methods

Blood samples. Peripheral blood samples were obtained before the onset of chemotherapy and upon complete remission after complete restoration of hematopoiesis (50-70 days after chemotherapy). Serum was collected and stored at -80 °C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation.

Cell lines and reagents. Chronic myelogenous leukemia K562 cells (ATCC) were cultured in IMDM (Gibco) containing 4 mM *L*-glutamine, 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS, from PAA) and 100 U/mL penicillin plus 100 µg/mL streptomycin (Gibco). Mouse AML C1498 cells was purchased from ATCC and C1498 cells expressing CRT on the cell surface (C1498.CRT) were generated as described previously (21). C1498 cells were cultured in DMEM (Gibco) containing 4 mM *L*-glutamine, 4.5 g/L glucose and supplemented with 10% FBS, 100 U/mL penicillin plus 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol (Gibco). All cell lines were regularly tested for *Mycoplasma* by using MycoAlert® Mycoplasma Detection Kit (Lonza) and Spark reader (Tecan) detecting luminescence. The cell lines were used for experiments after 2 to 5 passages from thawing. Recombinant human calreticulin (rCRT, from Sino Biological Inc.) was added to either NK cell or whole PBMC cultures from heathy donors (HDs) to a final concentration of 5 µg/mL. Cells were incubated with rCRT overnight at 37 °C before analysis of cell phenotype and function by flow cytometry.

Isolation of NK cells. NK cells were isolated from fresh PBMCs from AML patients before the initiation of induction chemotherapy (n=10) and upon complete remission and complete restoration of hematopoiesis (n=10) by negative selection with the NK cell isolation kit

(Miltenyi Biotec) according to the manufacturer's instructions. The purity of NK cells isolated from AML patients at diagnosis was increased by two-step pre-purification procedure including the exclusion of $CD33^+$ malignant blasts, achieved with the EasySep human CD33 positive selection kit (STEMCELL Technologies), and the exclusion of $CD34^+$ blasts, based on the CD34 MicroBead kit (Miltenyi Biotec). Freshly isolated NK cells were labeled with the Vybrant DiI cell labeling solution (Invitrogen) and maintained overnight in RPMI 1640 medium (Gibco) containing 10% AB human serum (Invitrogen) and supplemented with 200 U/mL IL-2 (PeproTech) for pre-activation. Alternatively, NK cells were isolated from PBMCs from HDs after overnight incubation with 5 μg/ml rCRT (Sino Biological Inc.) and used for subsequent cytotoxicity assay.

NK cell cytotoxicity assay. NK cells from AML patients or HDs were tested for their capacity to kill NK cell-sensitive K562 cells. NK cells labeled with Vybrant dye DiI were co-cultured with target DiD-labeled K562 cells in RPMI 1640 medium containing 10% AB human serum in 96-well U-bottom plates (Nunc) at two different effector:target cell ratios (10:1 and 5:1). After 4 h, cells were harvested, washed by PBS and co-stained with Annexin V and DAPI. Viability of target DiD^+ K562 cells was determined by flow cytometry.

Degranulation and IFN-γ production after *in vitro* stimulation. To assess NK cell and T cell function in AML patients, fresh PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, from Sigma Aldrich) plus 1 μg/mL ionomycin or with K562 cells at PBMC:tumor cell ratio 10:1 plus overnight incubation with 200 U/mL IL-2 in the presence of anti-CD107a-FITC monoclonal antibody (BioLegend) for 1 h, followed by 3 h incubation with brefeldin A (BioLegend). Cells were then washed in PBS, stained with anti-CD45-PE (EXBIO), anti-CD3-AlexaFluor700 (EXBIO), anti-CD4-ECD (Beckman Coulter), anti-CD8-HV500 (BD Biosciences) and anti-CD56-PerCP-Cy5.5 monoclonal antibodies (BioLegend), fixed in fixation/permeabilization buffer (eBioscience), further permeabilized with permeabilization

buffer (eBioscience) and intracellularly stained with anti-IFN-γ-PE-Cy7 (eBioscience), anti-granzyme B-BrilliantViolet421 (BD Biosciences) and anti-perforin-APC monoclonal antibodies (BioLegend). We also determined NK cell and T cell function in the spleen and/or tumors in C57BL/6 (B6) mice after C1498.WT/CRT cell injection. Fresh mouse splenocytes and/or tumor cell suspension were stimulated with PMA plus Ionomycin (to assess NK cell function) and with PMA plus Ionomycin or Dynabeads Mouse T-Activator CD3/CD28 beads at bead-to-cell ratio of 1:1 (Life Technologies) (to assess T cell function) and stained with anti-mouse antibodies following the same protocol as described above. Alternatively, PBMCs or NK cells isolated from HDs were pre-incubated with 5 μg/mL rCRT overnight at 37 °C and stimulated and processed as described above.

Mice. C57BL/6 (B6) mice were obtained from the animal facility of the Institute of Physiology (Czech Academy of Sciences, CAS), v.v.i.. Mice were maintained in the conventional animal facility of Institute of Microbiology of CAS, v.v.i. and used for experiments when at 9-15 weeks of age. Mice were regularly screened for mouse hepatitis virus (MHV) and other common mouse pathogens, according to FELASA guidelines. All experiments were approved by the Animal Welfare Committee at the Institute of Microbiology of CAS, v.v.i.. To generate tumors *in vivo*, $1x10^6$ C1498.WT or C1498.CRT cells were inoculated *s.c.* into the lower right flank of B6 mice in 100 μL of PBS on day 0 (D0). Tumor size was measured every 2 days by standard laboratory caliper and determined according to the following formula: size= (length x weight) mm^2. Mice were sacrificed on D3 or D19 and spleen and tumors were harvested for analysis of functional status of NK cells (spleen on D3) and T cells (both spleen and tumors on D19) by flow cytometry.

Circulating cytokine analysis in AML patients. The levels of IFN-γ, IFN-α2, IL-21, IL-15, IL-7 and IL-3 in the serum of patients with AML in complete remission (n=60) were determined by Luminex assay (custom made MILLIPLEX® MAP 6-Plex Human Cytokine Magnetic Bead

Panel Kit; Merck Millipore) according to the manufacturer's instructions. Data were acquired using Luminex MAGPIX.

Isolation of RNA from PBMCs and reverse transcription. Total RNA was isolated with the RNeasy Mini Kit (Qiagen). Cell lysate in RLT buffer enriched with 1% 2-mercaptoethanol was quickly thawed and processed as per manufacturer's instructions, including a DNase I digestion step. RNA concentration and purity were determined using a NanoDrop 2000c (Thermo Scientific), and RNA integrity was assessed using an Agilent 2000 Bioanalyzer (Agilent). Purified RNA samples were stored at -80 °C until further use. RNA samples with an integrity number ≥ 7 were used for reverse transcription. cDNA for the detection of selected 46 genes associated with immune system, including NK cell genes, was synthesized from 200 ng of total RNA using the TATAA GrandScript cDNA Synthesis Kit (TATAA Biocenter).

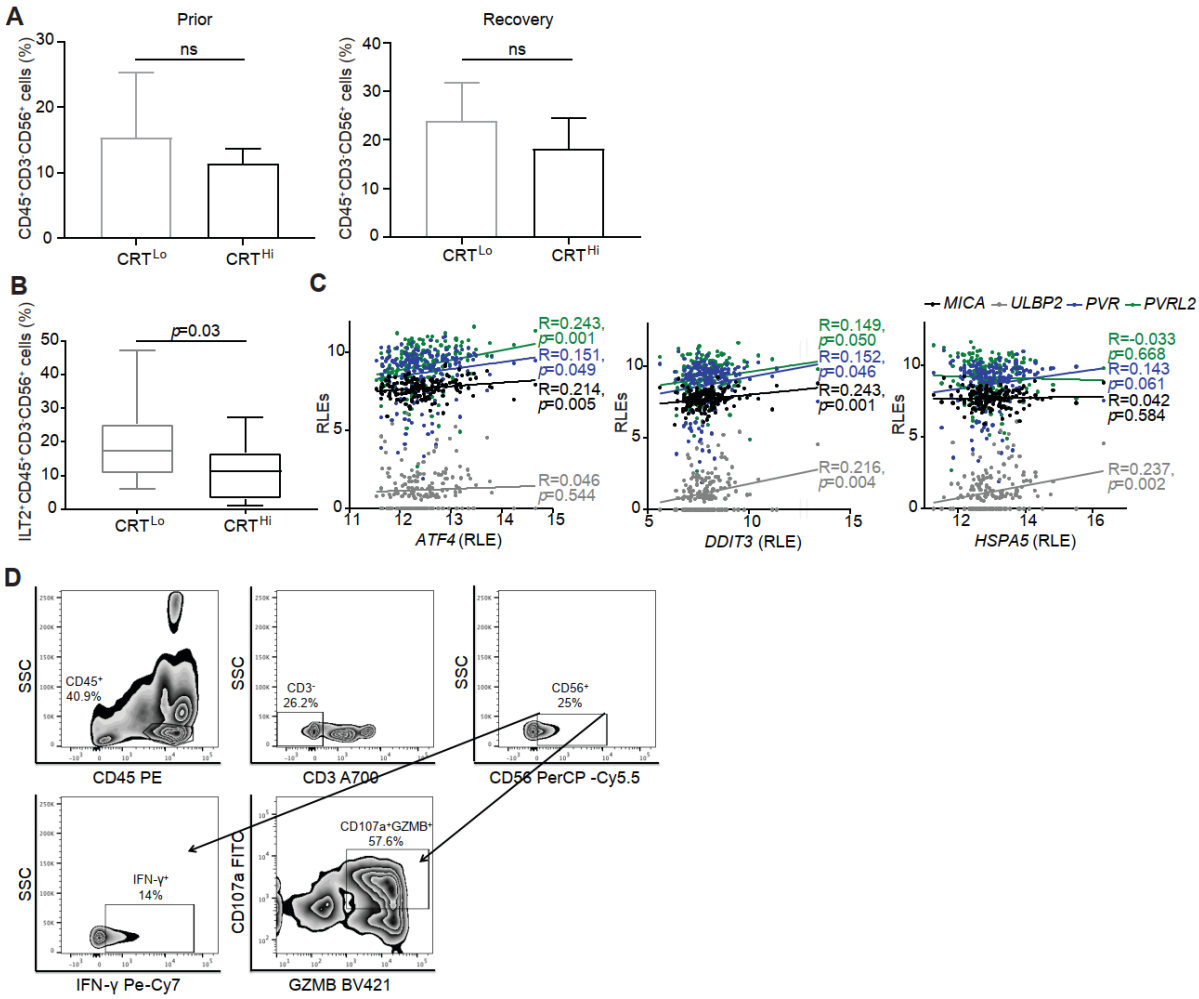
cDNA preamplification. Ten microliters of 2-times diluted cDNA were used in a 50 µL preamplification reaction using a TATAA PreAmp GrandMaster® Mix and primers at final concentration of 40 nM per primer. The following thermal profile for targeted pre-amplification was implemented on a T100 Thermal Cycler (Bio-Rad): 95 °C for 3 min, followed by 14 cycles of amplification (95 °C for 20 s, 55 °C for 3 min and 72 °C for 20 s). After the final extended (10 min) elongation step, samples were immediately frozen and stored at -20 °C until analysis.

High-throughput quantitative real-time PCR. High-throughput qPCR was performed on the BioMark system using the 48.48 Dynamic Array Chip for Gene Expression and probe-based detection. Each 5 µL sample reaction contained 1 µL of 10-times diluted pre-amplification product, 2.74 µL Probe GrandMaster Mix (TATAA Biocenter), 0.25 µL 20X GE Sample Loading Reagent (Fluidigm), 0.01 µL ROX (Life Technologies, the final concentration in the sample mix was 50 nM) and DNA/DNAse-free water. The 5 µL assay reaction mix contained 2.5 µL Assay Loading Reagent (Fluidigm) and 2.5 µL of a 5 µM mix of reverse and forward

primers plus 2.5 μM probes. Priming and loading of the dynamic array were performed according to the manufacturer´s instructions using the IFC controller HX. The temperature profile was thermal mixing at 50 °C for 2 min followed by 70 °C for 40 min and 25 °C for 10 min, hot start activation at 95 °C for 30 s and 40 cycles of amplification (95 °C for 10 s and 60 °C for 60 s). Melting curve analysis was performed in the range of 60 °C to 95 °C at 0.5 °C per second increments. Amplification data were analyzed with Fluidigm Real-Time PCR Analysis software, applying the linear derivative baseline subtraction method and a user-defined global threshold to obtain Cq values. Melting curve analysis was performed on all samples. Pre-amplification and Biomark´s measurement were performed at BIOCEV Gene Core Facility (Vestec, Prague).

Supplemental Figures

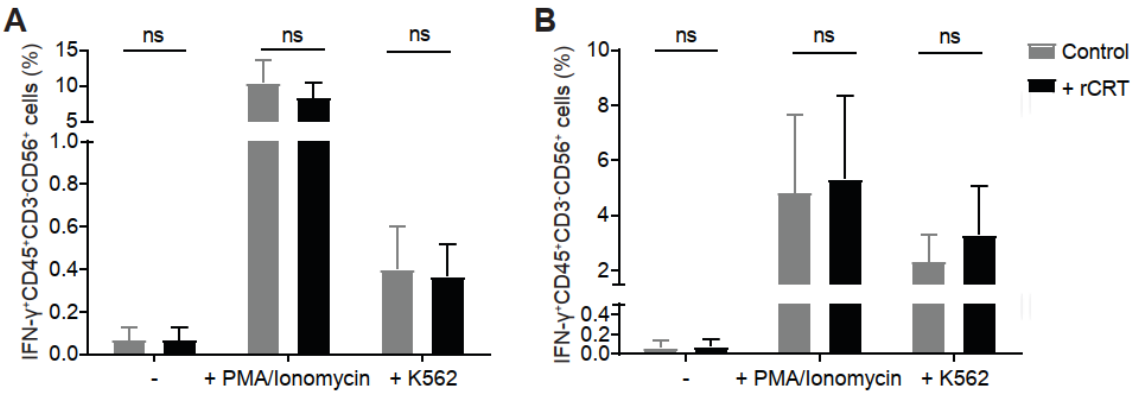
Supplemental Figure 1



(A) The percentage bone-marrow $CD45^{+}CD3^{-}CD56^{+}$ NK cells in CRT^{Hi} *versus* CRT^{Lo} AML patients before the induction chemotherapy (Prior, n=45) and at re-establishment of normal hematopoiesis (Recovery, n=10) determined by flow cytometry. Boxplots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns, not significant. **(B)** The frequency of $CD45^{+}CD3^{-}CD56^{+}$ NK cells staining positively for inhibitory NK cell receptor ILT2 in CRT^{Hi} and CRT^{Lo} AML patients upon complete remission and re-establishment of normal hematopoiesis (Recovery, n=31) determined by flow cytometry. Boxplots: lower quartile,

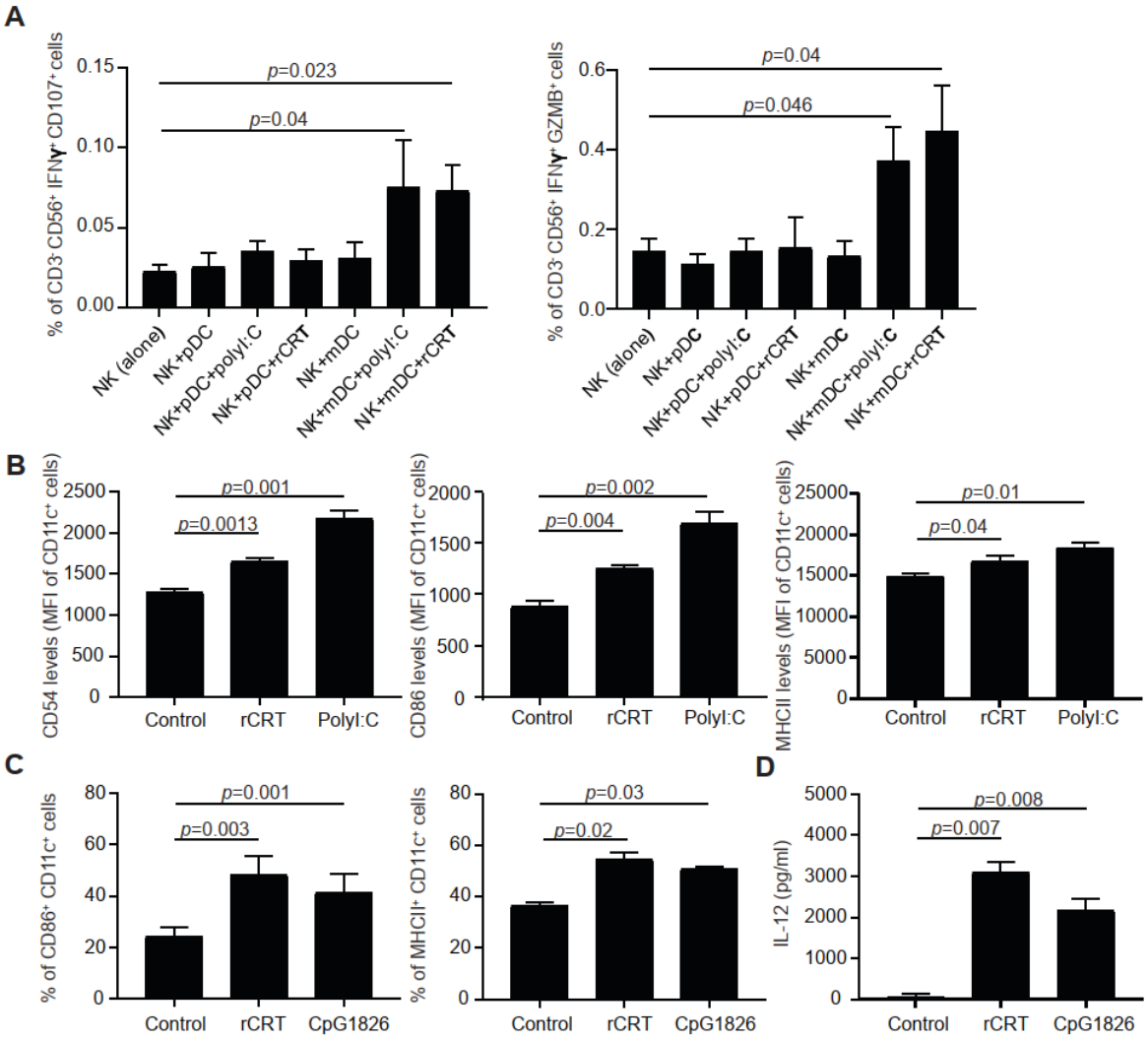
median, upper quartile; whiskers, minimum, maximum. **(C)** Linear regression analysis between NK cell ligand gene (*MICA*, *ULBP2*, *PVR*, *PVRL2*) mRNA levels and ER stress response gene (*ATF4*, *DDIT3* and *HSPA5*) mRNA levels in 173 AML patients from the TGCA public database. R, Pearson correlation coefficient; ns, not significant. **(D)** Gating strategy for NK cells and their functional properties. The percentage of cells in each gate is reported.

Supplemental Figure 2



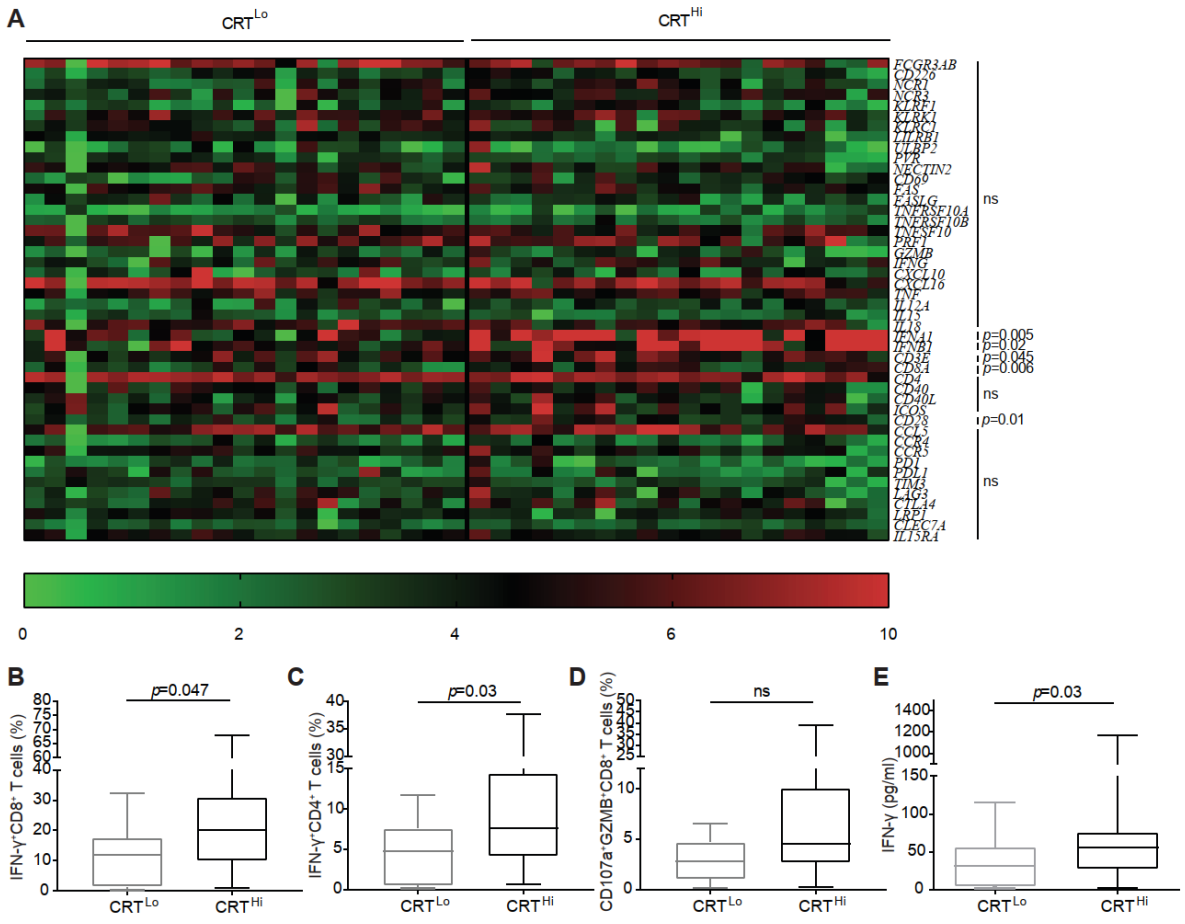
(A, B) The effect of recombinant human calreticulin (rCRT, Sino Biological Inc.) on effector functions of isolated HD NK cells (n=8) or NK cells in HD PBMC mixture (n=8). Purified NK cells **(A)** or whole PBMCs **(B)** were pre-incubated with 5 µg/ml of rCRT overnight and subsequently stimulated by PMA + Ionomycin or K562 cell line for 4 h. The percentage of responding (IFN-γ^{+}CD45^{+}CD3^{-}CD56^{+}) NK cells was determined by flow cytometry. NK cells/PBMCs without rCRT and unstimulated NK cells/PBMCs were used as a negative controls. ns, not significant.

Supplemental Figure 3



(A) Percentage of $CD3^{-}CD56^{+}IFN\gamma^{+}CD107^{+}$ and $CD3^{-}CD56^{+}IFN\gamma^{+}GZMB^{+}$ NK cells upon exposure to human myeloid DCs (mDCs) or plasmacytoid DCs (pDCs) activated by rCRT or polyI:C. **(B)** Expression levels of CD54, CD86 and MHC Class II molecules on $CD11c^{+}$ cells exposed to rCRT or polyI:C for 24 hours. **(C)** Percentage of $CD86^{+}$ or $MHCII^{+}$ cells amongst mouse bone marrow-derived $CD11c^{+}$ DCs upon cultivation with rCRT or CpG1826 for 24 hours. **(D)** Level of IL-12 secreted by mouse $CD11c^{+}$ bone marrow-derived DCs after cultivation with rCRT or CpG1826 for 24 hours determined by ELISA.

Supplemental Figure 4



(A) Quantitative RT-PCR-assisted quantification of the expression levels of 46 genes associated with immune system in PBMCs from 20 CRT^{Hi} *versus* 21 CRT^{Lo} AML patients at recovery of normal hematopoiesis. **(B, C)** The percentage of IFN-γ^+ $CD8^+$ and $CD4^+$ T cells and $CD107a^+/GZMB^+CD8^+$ T cells **(D)** upon PMA + Ionomycin stimulation in 13 CRT^{Lo} and 13 CRT^{Hi} AML patiens upon the restoration of normal hematopoiesis. Patient samples were analyzed by flow cytometry. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. ns, not significant. **(E)** The levels of IFN-γ in the sera of 28 CRT^{Hi} *versus* 29 CRT^{Lo} AML patients at recovery of normal hematopoiesis. The concentration of IFN-γ was determined by ELISA.

Supplemental tables

Supplemental Table 1. Anti-human antibodies used for flow cytometry.

Parameter	source	Producer	Clone	Fluorochrome	Dilution
B7-H6	mouse	R&D Systems	875001	PE	6:100
CCR7	mouse	BioLegend	G043H7	PerCP-Cy5.5	6:100
CD3	mouse	EXBIO	MEM-57	Alexa Fluor 700	5:100
CD3	mouse	BD Biosciences	SK7	AmCyan	6:100
CD4	mouse	eBioscience	RPA-T4	PE-Cy7	5:100
CD4	mouse	Beckman Coulter	SFCI12T4D11	ECD	5:100
CD8	mouse	BD Biosciences	RPA-T8	HV500	5:100
CD11c	mouse	BioLegend	3.9	Brilliant Violet 421	4:100
CD11c	mouse	EXBIO	BU15	APC	6:100
CD14	mouse	EXBIO	MEM-15	FITC	6:100
CD16	mouse	BioLegend	3G8	Pacific Blue	6:100
CD45	mouse	Life Technologies	HI30	PE-Texas Red	6:100
CD45	mouse	EXBIO	MEM-28	PerCP	6:100
CD45	mouse	BioLegend	HI30	Pacific Blue	6:100
CD45	mouse	EXBIO	MEM-28	PE	2:100
CD56	mouse	BioLegend	MEM-188	PerCP-Cy5.5	6:100
CD56	mouse	EXBIO	MEM-188	FITC	6:100
CD86	mouse	BD Biosciences	FUN-1	Alexa Fluor 700	6:100
CD107a	mouse	BioLegend	H4A3	FITC	8:100
CD112	mouse	R&D Systems	610603	Alexa Fluor 700	6:100
CD155	mouse	BioLegend	SKII.4	PE-Cy7	6:100
CD158a/h/g	mouse	eBioscience	HP-MA4	APC	6:100
CD158b1/b2, j	mouse	Beckman Coulter	GL183	PE	6:100
CD158e1	mouse	BioLegend	DX9	Brilliant Violet 421	6:100
DNAM-1	mouse	BioLegend	11A8	PE	6:100
Granzyme B	mouse	BD Biosciences	GB11	Brilliant Violet 421	4:100
HLA-DR	mouse	BD Biosciences	L243	PE-Cy7	6:100
IFN-γ	mouse	eBioscience	4S.B3	PE-Cy7	1:100
ILT2	mouse	BioLegend	GHI/75	PE-Cy7	6:100
IL-15Rα	mouse	BioLegend	JM7A4	PE	6:100
MICA/B	mouse	BioLegend	GD4	APC	1:100
NKG2A	mouse	R&D Systems	131411	Alexa Fluor 700	6:100

NKG2D	mouse	BioLegend	1D11	PE-Cy7	6:100
NKp30	mouse	BioLegend	P30-15	APC	6:100
NKp46	mouse	BioLegend	9E2	Alexa Fluor 700	6:100
NKp80	mouse	Miltenyi Biotec		FITC	6:100
Perforin	mouse	BioLegend	dG9	APC	4:100
ULBP 2/5/6	mouse	R&D Systems	165903	PerCP	4:100

Supplemental Table 2. Anti-mouse antibodies used for flow cytometry.

Parameter	source	Producer	Clone	Fluorochrome	Dilution
CD45	rat	eBioscience	30-F11	eFluor 506	4:100
CD3	armenian hamster	BioLegend	145-2C11	PerCP	4:100
CD3	armenian hamster	eBioscience	145-2C11	FITC	4:100
CD4	rat	eBioscience	RM4-5	eFluor 450	4:100
CD8a	rat	eBioscience	53-6.7	Alexa Fluor 700	4:100
NK1.1	mouse	eBioscience	PK136	PE-Cy7	4:100
CD107a	rat	eBioscience	1D4B	PErCP-eFluor 710	4:100
CD107a	rat	eBioscience	1D4B	eFluor 450	4:100
IFN-γ	rat	eBioscience	XMG1.2	PE	4:100
Perforin	rat	eBioscience	eBioOMAK-D	APC	4:100
Granzyme B	rat	eBioscience	NGZB	PE-Cy7	4:100
CD86	rat	BD Bioscience	GL-1	PE	1:100
I-A(b) MHCII	armenian hamster	BD Bioscience	N418	BV421	1:100

Supplemental Table 3. Immune system-related genes evaluated by quantitative RT-PCR in AML patients upon restoration of normal hematopoiesis.

NK receptors	NK ligands	Inhibitory molecules	T cells	DC receptors	Cytokines	Cytotoxicity
FCGR3AB	*ULBP2*	*PD1*	*CD3E*	*LRP1*	*IL12A*	*CD69*
CD226	*PVR*	*PDL1*	*CD8A*	*CLEC7A*	*IL15*	*FAS*
NCR1	*NECTIN2*	*TIM3*	*CD4*	*IL15RA*	*IL18*	*FASLG*
NCR3		*LAG3*	*CD40*		*IFNA1*	*TNFRSF10A*
KLRF1		*CTLA4*	*CD40L*		*IFNB1*	*TNFRSF10B*
KLRK1			*ICOS*			*TNFSF10*
KLRC1			*CD28*			*PRF1*
LILRB1			*CCL5*			*GZMB*
			CCR4			*IFNG*
			CCR5			*CXCL10*
						CXCL16
						TNF