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ORIGINAL RESEARCH

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CEBPA mutants down-regulate AML cell susceptibility to NK-mediated lysis by disruption of the expression of NKG2D ligands, which can be restored by LSD1 inhibition

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ABSTRACT

NK group 2, member D (NKG2D) is one of the most critical activating receptors expressed by natural killer (NK) cells. There is growing evidence that acute myeloid leukemia (AML) cells may evade NK cell-mediated cell lysis by expressing low or no ligands for NKG2D (NKG2D-Ls). We hypothesized that CCAAT/enhancer-binding protein α (C/EBPα), one of the most studied lineage-specific transcription factors in hematopoiesis, might influence the expression of NKG2D-Ls. To test this hypothesis, we first examined the endogenous expression of wild-type C/EBPα (C/EBPα-p42) in human AML cell lines and demonstrated that its expression level was highly relevant to the sensitivity of AML cells to NK cell cytotoxicity. Induction of C/EBPα-p42 in the low endogenous *CEBPA*-expressing AML cell line increased the sensitivity to NK-induced lysis. Moreover, decreased expression of C/EBPα-p42 by RNA interference in AML cells abrogated NK-mediated cytotoxicity. We further showed that the increase in NK susceptibility caused by C/EBPα-p42 occurred through up-regulation of the NKG2D-Ls ULBP2/5/6 in AML cells. More importantly, chromatin immunoprecipitation (ChIP) coupled with high-throughput sequencing captured C/EBPα motif signatures at the enhancer regions of the *ULBP 2/5/6* genes. Whilst, the AML-associated C/EBPα C-terminal mutant and N-terminal truncated mutant (C/EBPα-p30) diminished *ULBP2/5/6* transcription. Finally, we identified that histone demethylase lysine-specific demethylase 1 (LSD1) inhibition can restore the expression of ULBPs via induction of *CEBPA* expression in AML cells, which may represent a novel therapeutic strategy for *CEBPA*-mutated AML.

Abbreviations: C/EBPα: CCAAT/enhancer-binding protein α; TF: Transcription factor; AML: Acute myeloid leukemia; TAD: Transactivation domain; FS: Frameshift; NK: Natural killer; NKG2D: NK group 2, member D; NKG2D-Ls: Ligands for NKG2D; MHC: Major histocompatibility complex; MICA: MHC class I-related chain A; ULBP: UL16-binding protein; STAT3: Signal transducer and activator of transcription 3; LSD1: Lysine-specific demethylase 1; Ab: Antibody; PBMC: Peripheral blood mononuclear cell; PBS: Phosphate-buffered saline; CFSE: Carboxyfluorescein diacetate succinimidyl ester; PI: Propidium iodide; shRNA: Short hairpin RNA; ChIP: Chromatin immunoprecipitation; BM: Binding motif; HCNE: Highly conserved noncoding element; TSS: Transcription start site; HMA: Hypomethylating agent; AZA: Azacitidine/5-aza-cytidine; DAC: Decitabine/5-aza-29-deoxycytidine; 2-PCPA: Tranylcypromine; RBP: RNA-binding protein; MSI2: MUSASHI-2; HDACi: Inhibitor of histone deacetylases; VPA: Valproate; DNMTi: DNA methyl transferase inhibitor; SCLC: Small cell lung cancer

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Background

CCAAT/enhancer-binding protein α (C/EBPα) is one of the most studied lineage-specific transcription factors (TFs) in hematopoiesis, which is mainly involved in cell fate decisions, including a key role in governing myeloid differentiation.[1] In line with the critical role of C/EBPα in granulocytic differentiation, the *CEBPA* gene was found to be mutated in approximately 7% of all patients with acute myeloid leukemia (AML), and the incidence increased to 10–15% in AML patients with normal karyotype.[2] Full-length C/EBPα is a 42-kDa protein (C/EBPα-p42) that contains two transactivation domains (TAD, TAD1 and TAD2) in the amino terminus and a basic leucine zipper domain (bZIP) at its carboxy terminus for DNA binding. Two major categories of collaborating *CEBPA* mutations have been described in human AML: (1) frameshift (FS) insertions or deletions affecting the N-terminal region resulting in

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a loss of the 42-kDa protein and overexpression of a shorter 30-kDa isoform (C/EBPα-p30), proposed to exhibit a dominant negative activity, and (2) in-frame mutations in the C-terminal region that alter the basic leucine zipper domain, leading to impaired DNA binding and disrupted protein–protein interactions.[3] Furthermore, it has been shown that nearly 10% of AMLs with biallelic mutations in *CEBPA* also harbor a germline *CEBPA* mutation.[4] These germline mutations are commonly additional C-terminal *CEBPA* mutations.[5,6] So *CEBPA* mutations may be clustered to germline mutations, N-terminal frameshift mutations and C-terminal mutations.

Natural killer (NK) cells play crucial roles as first-line defenders in the host response to tumors and infections. In contrast to T cells, NK cells elicit rapid anti-tumor responses based on signals from activating and inhibitory cell surface receptors. NK group 2, member D (NKG2D) is one of the most critical activating receptors expressed by NK cells.[7] Ligands for NKG2D (NKG2D-Ls) have been identified in humans, including major histocompatibility complex (MHC) class I-related chain A (MICA), MICB and UL16-binding proteins (ULBPs) 1/2/3/4/5/6. Several studies have shown that AML blasts, including AML stem cells, may also evade NK cell-mediated killing by expressing low or no NKG2D-Ls.[8,9] The regulation of expression of NKG2D-Ls remains only partially understood, while proteins involved in leukemogenesis, including p53,[10] c-Myc[11] or signal transducer and activator of transcription 3 (STAT3),[12] have been reported to play a regulatory role at the transcriptional or posttranscriptional level.

Published studies from multiple laboratories have contributed immensely to our understanding of how different *CEBPA* mutations disturb C/EBPα functions, including orchestrating granulopoiesis and leukemic transformation in AML.[13] Given its potential to bind to promoters or enhancers of multiple targeted genes as a TF, we questioned whether C/EBPα and its mutants disrupt the expression of NKG2D-Ls in AML cells and mediate their immune evasion. In the present study, we showed that wild-type C/EBPα (C/EBPα-p42) can increase AML susceptibility to NK-mediated cell lysis by up-regulation of the expression of ULBP 2/5/6 in AML cells. Functionally, we captured C/EBPα motif signatures at the enhancer regions of the *ULBP 2/5/6* genes. Whereas the AML-associated somatic mutants, C-terminal mutant and C/EBPα-p30, exhibit a loss or decrease in the ability to bind to these sites, both significantly decrease ULBP 2/5/6 expression in AML cells. Meanwhile, the *CEBPA* germline mutant retains the accessibility to the enhancer regions of the *ULBP 2/5/6* genes. More importantly, we identified that a histone demethylase lysine-specific demethylase 1 (LSD1) inhibitor, tranylcypromine (2-PCPA) hydrochloride, can reactivate C/EBPα expression and restore the expression of ULBPs in AML cells with low expression of C/EBPα-p42.

Materials and methods

Cell lines, primary AML samples and cell culture

Human myeloid leukemia cell lines (HL-60, THP-1, U937, NB4, KG-1) and HEK293T cells were routinely cultured in our lab. Bone marrow mononuclear cells were isolated from 10 *de novo* AML patients before chemotherapy and from healthy volunteers at Sir Run Run Shaw Hospital of the Zhejiang University School of Medicine. Primary human AML samples were obtained from bone marrow of AML patients with ≥70% blasts among mononuclear cells. The bone marrow mononuclear cells from AML patients and from healthy volunteers were isolated by density gradient centrifugation, viably frozen and freshly thawed for each experiment.

Approval for these studies was obtained from the ethics review committees of the First Affiliated Hospital of Zhejiang University School of Medicine and Sir Run Run Shaw Hospital of Zhejiang University School of Medicine. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Antibodies (Abs), reagents and small-molecule drugs

The following Alexa Flour 488-, PE- or APC-conjugated monoclonal antibodies (mAbs) were obtained from R&D Systems (Minneapolis, MN, USA), BioLegend (San Diego, CA, USA) or BD Bioscience (San Diego, CA, USA): anti-MICA/B, anti-ULBP1, anti-ULBP3, anti-ULBP4, anti-ULBP 2/5/6, anti-CD56, anti-CD107a and anti-CD3. Because of the high similarity of the ectodomains of ULBP 2, 5, 6, these three ULBP family members were detected by the same antibody.

The Epigenetics Compound Library containing 932 compounds was purchased from Targetmol (Boston, MA, USA).

Selection and expansion of NK cells

Primary NK cells were purified from peripheral blood mononuclear cells (PBMCs) of healthy human volunteers. PBMCs were isolated from whole blood by a density gradient with Ficoll-Paque Plus (GE Health Care, Piscataway, NJ, USA), followed by cultivation and expansion of NK cells using NK MACS® Basal Medium (Miltenyi Biotec, Germany) supplemented with 10% heat-inactivated human AB blood-type serum (Gemini, Woodland, California, USA) and cytokines IL-2 (500 IU/mL) and IL-15 (140 IU/mL) (Peperotech, Inc, NJ, USA) under standard culture conditions (a humidified 5% CO_2 atmosphere, 37°C) for 14 days (long-term culture). The initial seeding density of PBMCs was a median of 1×10^6 PBMCs in 6-well plates. After 7 days, the cells were transferred into T25 flasks. Fresh culture medium containing 10% human serum and cytokines was added every 2–3 days. Following 14 days of culture, purification of NK cells was performed using an NK cell isolation kit (Miltenyi Biotec, Germany), and about 90% of the cells were $CD56^+CD3^-$ as determined by flow cytometry. The cell viability exceeded 95%, as determined by trypan blue exclusion. NK cells used in each experiment were from the same volunteer. The experiment was repeated more than three times (with more than three different donors to avoid inter-donor variability).

NK cell cytotoxicity assay

We used purified NK cells as the effector cells and myeloid leukemia cell lines (HL-60, THP-1, U937, NB4, KG-1) as the target cells. Target cells were collected and rinsed twice with

phosphate-buffered saline (PBS), then incubated with 2 μM/L carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C for 10 min and gently mixed every 5 min. After that, ice cold PBS was added to the cells, which remained at 4°C for 5 min followed by centrifugation at 300 × g for 5 min; they were then rinsed twice and re-suspended with cold RPMI 1640 medium. Further, 1×10^5 labeled leukemic cells were co-cultured with 10×10^5 NK cells (E:T ratio 10:1) at 37°C in the dark for 4 h. At the end of the incubation time, the total contents of the U-bottom plates were transferred to Falcon tubes. The tubes were then put on ice and incubated with 2 μL of 4 μM propidium iodide (PI) for 10 min, followed by flow cytometric analysis within 1 h. Dead cells from the negative control (target cells incubated in the absence of effector cells) were considered to have undergone spontaneous lysis (basal lysis). Target cells killed by NK cells were measured as following formula: Specific lysis = [100× (% Sample lysis -% Basal lysis)] / (100 - Basal lysis),[14,15] in which sample lysis was defined as the CFSE and PI double-positive cells ($CFSE^+PI^+$) divided by the total target cells ($CFSE^+$) in the co-cultured cells, and basal lysis was the same ratio from the negative control.

NK degranulation assay

NK cells were co-cultured with target cells for 4 h at a 2:1 E:T ratio. Degranulation of NK cells was analyzed by flow cytometric analysis of CD107a expression.

Plasmids and lentivirus infection

In the present study, we chose our previously identified typical *CEBPA* mutants[16] for further evaluation. The GFP-tagged ORF clone of the *Homo sapiens* full-length *CEBPA* coding sequence (*CEBPA*, 1077 bp, NM_004364.2) (hereafter called pCMV6-AC-GFP-*CEBPA*-p42) was purchased from ORIGENE Technologies (Beijing, China). We used pCMV6-AC-GFP-*CEBPA*-p42 to generate the three typical *CEBPA* mutants by PCR: the germline mutant (584_589dup) (hereafter called *CEBPA*-G^m), the C-terminal in-frame mutant (914_916dup) (hereafter *CEBPA*-C^m) and the N-terminal truncated mutant represented by the p30 isoform (C/EBPα-p30). The wild-type C/EBPα expression vector and the three mutants were further subcloned into Flag-tagged p3× FLAG-Myc-CMV (Addgene, Watertown, MA, USA) and pHIV7/SFFV-GFP lentiviral plasmids (a gift from Prof. Jiing-Kuan Yee, City of Hope National Medical Center).

Detailed methods of lentivirus infection have been described previously.[17]

Lentiviral transduction and knockdown of *CEBPA* in AML cells

Recombinant lentiviral particles were produced in HEK293T cells by co-transfecting pGLV3/H1/GFP&Puro lentiviral plasmid containing the *CEBPA* short hairpin RNA (shRNA) sequence along with the packaging plasmids (PSPAX2 and PMD2.G). The virus-containing medium was harvested at 48–72 h after transfection and filtered using a 0.45 μm filter. For infection, the collected virus medium was added to the *CEBPA*-expressing cells. The cells were selected as GFP-positive by flow cytometry. *CEBPA* shRNA was induced by polybrene, and the C/EBPα protein level was analyzed by immunoblot. The *CEBPA* shRNA sequence comprises sequence 1: 5′-GCCAAGAAGTCGGT GGACAAG-3′, and sequence 2: 5′-CAACGTGGAGACG CAACAGAA-3′.

Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) and ChIP-qPCR

For the chromatin immunoprecipitation (ChIP) assay, 1×10^7 HEK293T cells transfected with wild-type C/EBPα expression vector (p3× FLAG-Myc-CMV- *CEBPA*-p42) or empty vector were harvested and processed with the SimpleChiP® Enzymatic Chromatin IP Kit (Agarose Beads) (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Immunoprecipitation was performed with rabbit anti-FLAG® M2 (14793, Cell Signaling Technology), anti-histone H3 (D2B12) XP® (Cell Signaling Technology, #4620) and IgG control antibodies (Cell Signaling Technology, # 2729). The antibody-bound chromatin was captured with Protein-G sepharose beads, washed, de-cross-linked and precipitated. Libraries were sequenced following the protocol provided by the I NEXTFLEX® ChIP-Seq Library Prep Kit for Illumina® Sequencing (NOVA-5143, Bioo Scientific, Austin, TX) and sequenced on Illumina Xten using the PE 150 method. Sequencing reads were mapped to the reference genome assembly (hg18) using Bwa (version 0.7.15), and peak calling was performed using MACS2 software (version 2.1.1.20160309).

The relative amounts of chromatin immunoprecipitated by either isotype control or FLAG mAb were quantified by real-time PCR with specific primers for the C/EBPα binding sites in *ULBP* gene promoters or enhancers, respectively. Sequences of primers used for ChIP-qPCR are listed in Supplementary Table 1.

Luciferase reporter assays

For the luciferase assays, HEK293T cells at 50% confluency were transfected in 24-well plates with *ULBP* promoter luciferase reporter (pGL3 ULBPs) or empty-vector control (pGL3), with pCMV6-AC-GFP-*CEBPA*-p42 expression plasmid or pCMV6-AC-GFP empty vector, together with pRL-TK Renilla (Promega, Fitchburg, WI, USA). After 48 h, cells were lysed and luciferase activity was assessed using a Dual-Luciferase-Reporter Assay System (Promega, USA).

We also constructed a psiCHECK2 plasmid, which is mutated at binding sequence of C/EBPα in the *ULBP* genes, to confirm C/EBPα motif signatures at enhancer regions of the *ULBP* genes. The psiCHECK2 plasmid is simultaneously expressing *ULBP* promoter luciferase reporter and Renilla luciferase reference. Assays were performed in at least three independent experiments.

Quantitative real-time PCR

Total RNA was extracted from cell samples by TRIzol solution (Invitrogen), and cDNA was transcribed with a reverse transcription kit (Vazyme, Nanjing, China) in accordance with the manufacturer's protocols. PCR reactions were performed using

a quantitative PCR kit (Vazyme) and fluorogenic quantitative PCR instrument (Bio-Rad). Sequences of primers used for RT-qPCR are listed in Supplementary Table 2. The amount of the target gene was normalized to that of the housekeeping gene *GAPDH*. All data were presented as log ratio of the target gene/ *GAPDH*.

Western blot

Western blot analysis was performed as previously described[17] using whole cell lysates with the following primary antibodies: rabbit anti-C/EBPα (1:200, Abcam), FLAG (1:1000, Sigma-Aldrich)or β-tubulin(1:1000, TransGen) and GAPDH (1:1000, Santa Cruz). The membranes were then washed with PBST and incubated with goat anti-mouse or anti-rabbit-HRP secondary antibody (1:5000, Santa Cruz Biotechnology).

Flow cytometry analysis

Expression of surface markers on AML cells or on NK cells were determined by flow cytometry analysis. Cells were collected, washed twice with PBS containing 2% FBS, stained for 30 min at 4°C with labeled Abs according to manufacturers' recommendations. Flow cytometry analysis was performed using a flow cytometer (Beckman Coulter, Inc., Miami, FL, USA), and isotype antibodies were used to assess nonspecific staining. Data were processed using Flowjo V10.7.1. The results were presented as rate of positive cells and/or median fluorescene intensity (MFI). Quantification of the expression level of surface molecules on AML cells or on NK cells was evaluated using the MFI/isotype control MFI.

Xenograft model studies

All animal experiments were conducted in immune-deficient NOD/ShiLtJGpt-Prkdc^{em26}/IL2rg ^{em26}/Gpt (NCG) mice originally purchased from GemPharmatech (Jiangsu, China). Mice were housed and bred in Zhejiang Academy of Medical Sciences Central Animal Laboratory and were used in experiments at 5 to 6 weeks of age. Studies were approved by the Animal Experimental Committee of Zhejiang University School of Medicine. NB4 cells were engineered to express the green fluorescent protein (GFP) and luciferase reporter genes (NB4.LucGFP cells) for longitudinal tumor load monitoring by bioluminescence imaging. The preclinical xenograft model for AML was established by intravenous injection of 2×10^5 NB4. LucGFP cells through the tail vein (i.v. on day 0). In adoptive transfer studies, 1.2×10^6 primary NK cells were injected intravenously through the tail vein (i.v. on day +1). Based on efficacious compounds screened from the Epigenetics Compound Library, the experimental designs implemented for the treatment of mice were intraperitoneal injection of small-molecule inhibitors (on day +2).

Statistical analyses

Data were expressed as the mean ± SD of at least three independent experiments, and each group had three repetitions. Different groups were compared by using Independent-Samples T tests and Fisher's exact tests. Statistical analyses were performed using SPSS software version 16.0. All probability values were generated from two-sided tests. *P < .05; **P < .01, and ***P < .001.

Results

Correlation of the endogenous expression of wild-type C/EBPα in AML cells with sensitivity to NK cell cytolysis

We first investigated the endogenous expression of wild-type C/EBPα (C/EBPα-p42) in five human AML cell lines. RT-qPCR and Western blotting analysis showed that endogenous C/EBPα-p42 expression in the HL-60, THP-1 and U937 cell lines was significantly higher than that in the NB4 and KG-1 cell lines (Figure 1(a,b)). The five AML cell lines were further co-cultured with NK cells at an E:T ratio of 10:1. The NK cytotoxicity assay showed that the HL-60, THP-1 and U937 cells with higher endogenous C/EBPα-p42 expression were more prone to NK-cell-mediated killing than the lower endogenous C/EBPα-p42 expressing NB4 and KG-1 cells. The killing effects of NK cells on the HL-60, THP-1 and U937 cells were 60.72 ± 3.91%, 53.5 ± 2.7% and 57.16 ± 12.86%, respectively, which were greater than those on the NB4 (35.93 ± 3%) and KG-1 (26.96 ± 8.22%) cells (Figure 1(c)). Moreover, there were higher levels of degranulation of NK cells against HL-60 (33.47 ± 1.45%), THP-1(25.42 ± 1.87%) and U937 (31.43 ± 2.22%) targets compared to NB4 (18.79 ± 0.88%) and KG-1 (5.35 ± 0.61%) targets (Figure 1(d)).

Correlation analysis using the Pearson model revealed that the expression level of C/EBPα-p42 in the AML cell lines was highly relevant to the sensitivity of AML cells to NK cell cytotoxicity (Figure 1(e)) and effective induction of NK-cell activation (Figure 1(f)).

Induction of susceptibility of AML cells to NK cells by wild-type C/EBPα occurred through up-regulation of NKG2D ligands ULBP 2/5/6

To elucidate the mechanisms underlying the increased susceptibility to NK cells of C/EBPα-p42, we examined the expression pattern of NK cell receptor ligands in the AML cell lines by flow cytometry analysis. Our results showed that the expression of ULBP2/5/6 was significantly up-regulated on the surface of the HL-60 (34.9 ± 2.93%), THP-1 (71 ± 2.19%) and U937 (91.6 ± 0.87%) cell lines, which have higher endogenous C/EBPα-p42 expression, compared with the lower endogenous C/EBPα-p42 expressing NB4 (0.34 ± 0.08%) and KG-1 (2.82 ± 0.48%) cells ($P < .001$) (Figure 2(a)). The trend was similar by the method of median fluorescence index (MFI)/ isotype (Figure 2(a)). Meanwhile, the expression of MICA/B was not affected (Figure 2(b)).

We next examined the association of C/EBPα-p42 with the expression levels of ULBP2, 5 and 6 in primary AML cells from patients with *de novo* AML. Compared with healthy volunteers (n = 3), the mean mRNA expression of *CEBPA* in AML cells from those patients (n = 10) was lower as was the expression of *ULBP2, ULBP6* ($P < .05$) (Figure 2(c)). Furthermore, the mean protein expression of C/EBPα-p42

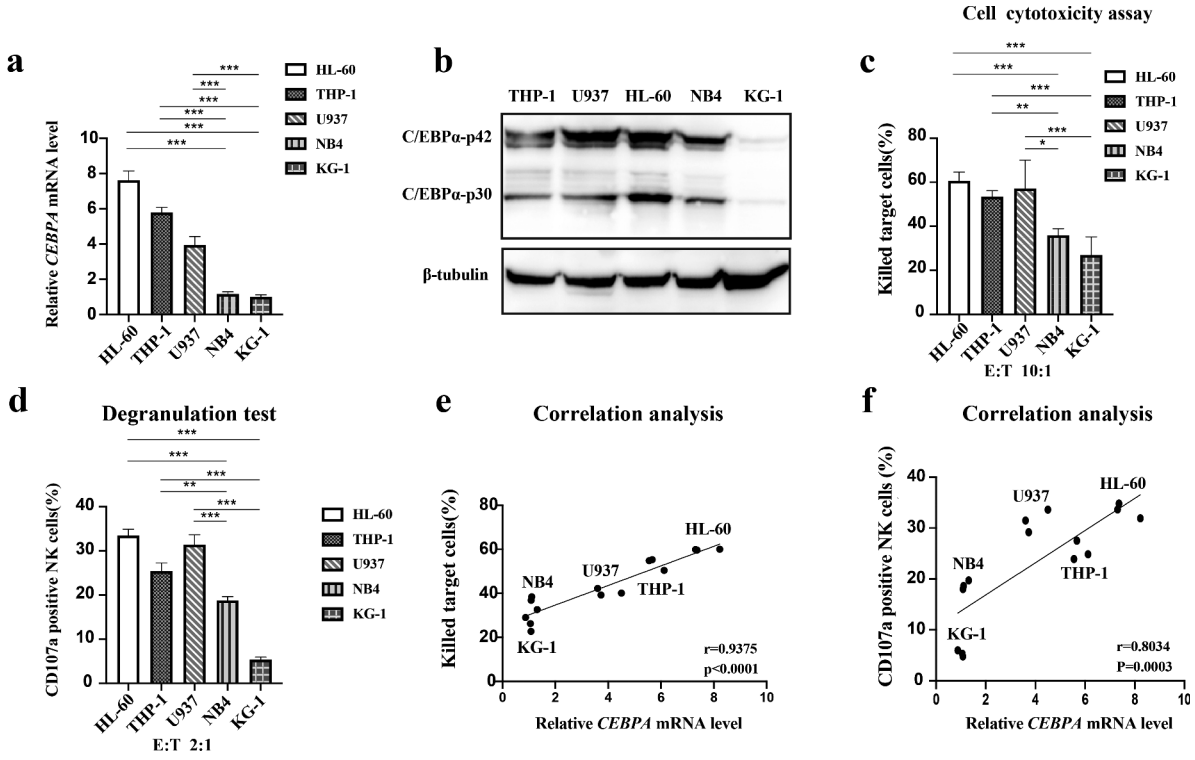

Figure 1. Correlation of the endogenous expression of wild-type C/EBPα in AML cells with sensitivity to NK cell cytolysis. (a-b). RT-qPCR (a) and Western blotting (b) analysis of endogenous C/EBPα-p42 expression in the AML cell lines, THP-1, U937, HL-60, NB4 and KG-1. (c). NK cytotoxicity assay of target cells (HL-60, THP-1, U937, NB4, KG-1) co-incubated with NK cells at an E:T ratio of 10:1. (d). Flow cytometric analysis of CD107a expression on NK cells after being co-incubated for 4 hours with AML cell lines (HL-60, THP-1, U937, NB4, KG-1) at an E:T ratio of 2:1 (representative blot of n = 3). (e–f). Correlation analysis using the Pearson model revealed that the expression level of *CEBPA* in AML cell lines was highly relevant to the sensitivity of AML cells to NK cell cytotoxicity (e) and effective induction of NK-cell activation (f). Data represent mean ± SD (n = 3); *P < .05; **P < .01, ***P < .001.

in primary AML cells from AML patients (n = 4) was lower than those from healthy volunteers (n = 5) ($P < .05$) (Figure 2(c)). We also performed correlation analysis using the Pearson model revealed that the expression levels of *CEBPA* in primary AML cells were highly relevant to the expression levels of *ULBP2* (r = 0.8493; $P < .0001$) and *ULBP6* (r = 0.8368; $P < .0001$) (Figure 2(c)). We further examined the relationship between *ULBPs* and *CEBPA* expression using publicly available human AML microarray data from GEO (https://www.ncbi.nlm.nih. gov/ geo). We searched gene expression profiling data of AML patients, which are available of status of *CEBPA* gene (wild type or mutant), gene expression profiling data of *CEBPA* and *ULBPs*. Gene expression profiling data from 91 patients with wild-type *CEBPA* were selected and the Pearson's correlation test was applied. A significant positive linear correlation was observed between *CEBPA* and *ULBP2* expression in these cases (r = 0.21; $P = .049$) (Supplementary Figure S1A). Furthermore, gene expression profiling data from 18 patients with mutated *CEBPA* were selected and the Pearson's correlation test was applied. An inverse correlation was observed between mutated *CEBPA* and *ULBP2* expression in these cases (r = – 0.611; $P = .007$). (Supplementary Figure S1B).

Next, we hypothesized that the inhibition of C/EBPα-p42 expression may affect ULBP2/5/6 expression. We chose the U937 cell line, a high endogenous C/EBPα-p42-expressing and NK-sensitive cell line, to knock down the *CEBPA* gene by shRNA lentiviral vector transfection. Positive single clones were chosen and confirmed by RT-qPCR and Western blotting analysis (Figure 2(d)). Compared with the negative control group, U937 cells with *CEBPA* knockdown (U937-shCEBPA-clone1, U937-shCEBPA-clone2) displayed a significantly reduced expression of ULBP2/5/6 (clone 1: 54.65 ± 2.8% vs. 74.12 ± 4.68%; P < .01; clone 2: 56.02 ± 4.66% vs. 74.12 ± 4.68%; $P < .01$) (Figure 2(e)) and reduced sensitivity to NK-induced lysis (clone 1: 50.6 ± 1.4% vs. 67.63 ± 0.38%; $P < .001$; clone 2: 52.57 ± 3.8% vs. 67.63 ± 0.38%; $P < .01$) (Figure 2(f)).

Taken together, these results suggest that C/EBPα-p42 is involved in the regulation of expression of ULBP 2/5/6 in AML cells.

Wild-type C/EBPα activates ULBP 2/5/6 transcription by directly binding to the enhancer regions of the ULBP genes

To get more insight into the mechanisms of up-regulation of the *ULBP* genes by C/EBPα-p42, we asked whether the transcriptional up-regulation of *ULBP2/5/6* involved C/EBPα binding motifs (C/EBPα BMs) in the promoter or enhancer regions of the *ULBP* genes. As shown in Figure 3(a), ChIP-seq revealed 7 C/EBPα-bound peaks in the *ULBP* genes in HEK293T cells. The seven putative C/EBPα BMs were located in highly conserved noncoding elements (HCNEs) of the *ULBP* genes assembly at chr 6.

To determine if the putative C/EBPα BMs were functional, the seven HCNEs, including the putative C/EBPα BM DNA fragment (100–200 bp up- and down-stream relative to the

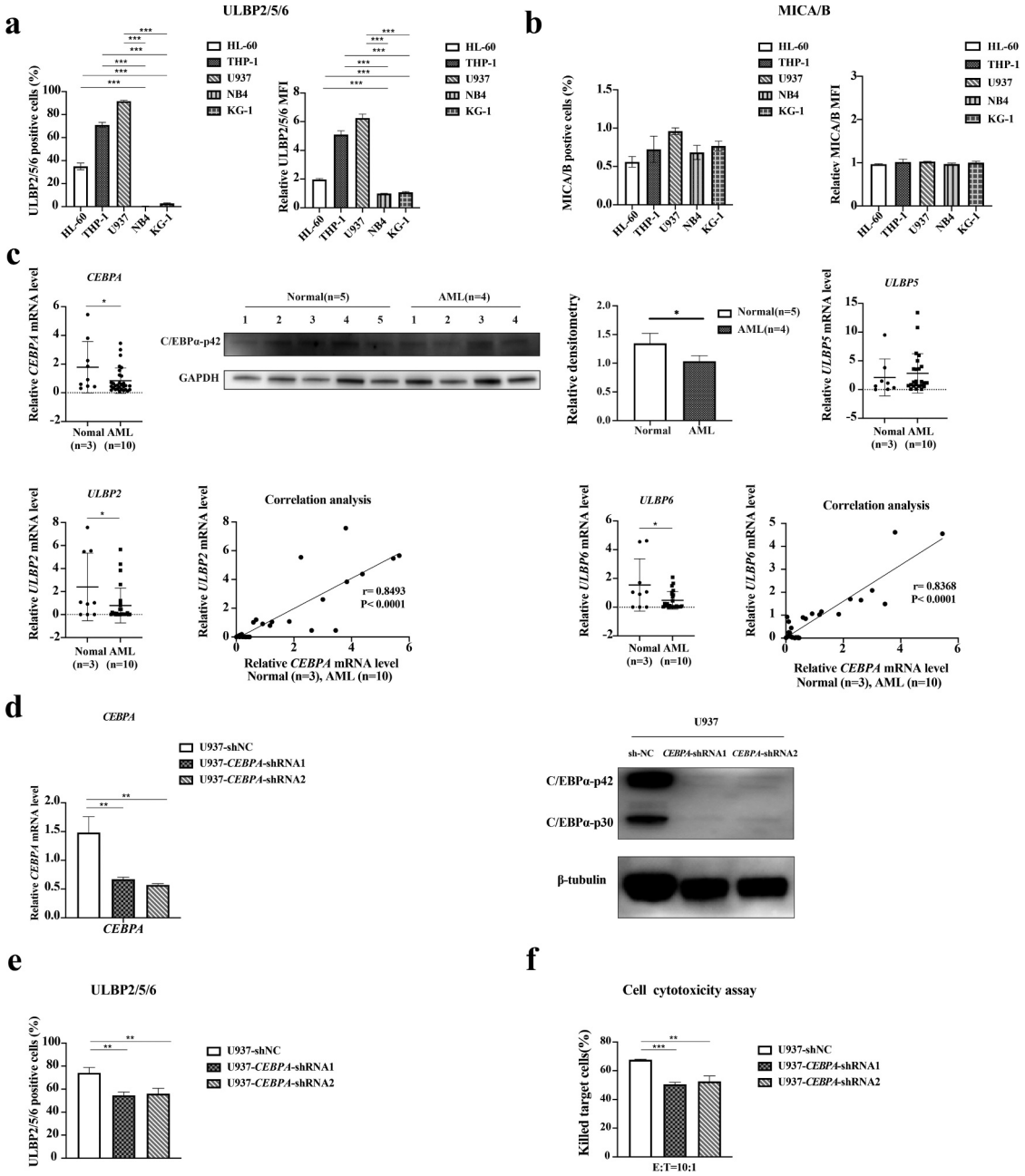

Figure 2. Association of the expression level of wild-type C/EBPα with the NKG2D ligands ULBP 2/5/6 in AML cells. (a). Quantification of the expression levels of ULBP 2/5/6 on AML cell lines by flow cytometry. Data were presented as rate of positive cells and median fluorescene intensity (MFI). Quantification of the expression level of surface molecules on AML cells was evaluated using the MFI/ isotype control MFI. (b). The expression levels of MICA/B on AML cell lines presented as rate of positive cells and MFI. (c).The top of the figure C: The relative *CEBPA* mRNA level in primary AML cells from patients with *de novo* AML (n = 10) compared with those of healthy volunteers (n = 3) ($P < .05$). The mean protein expression of C/EBPα-p42 in primary AML cells from AML patients (n = 4) was lower than that from healthy volunteers (n = 5) ($P < .05$). The relative *ULBP5* mRNA level in primary AML cells from patients with *de novo* AML (n = 10) was comparable to those of healthy volunteers (n = 3). The bottom of the figure C: The relative mRNA levels of *ULBP2* and *ULBP6* in primary AML cells from patients with *de novo* AML (n = 10) were significantly lower than those of healthy volunteers (n = 3) ($P < .05$).Correlation analysis using the Pearson model revealed that the expression levels of *CEBPA* in primary AML cells were highly relevant to the expression levels of *ULBP2* and *ULBP6*. (d). The *CEBPA* gene in U937, a high endogenous C/EBPα-p42 expressing and NK-sensitive cell line, was knocked down by shRNA lentiviral vector transfection. Positive single clones were chosen and confirmed by RT-qPCR (left) and Western blotting analysis (right). (e–f). Compared with the negative control group, U937 cells with *CEBPA* knockdown (U937-shCEBPA-clone1, U937-shCEBPA- clone2) displayed a significantly reduced expression of ULBP2/5/6 (e) and reduced sensitivity to NK-induced lysis (f). (Because of the high similarity of the ectodomains of ULBP2, 5, 6, these three ULBP family members were detected by the same antibody.).

respective sequence) on the *ULBP* locus were cloned into luciferase reporter plasmids and tested for activation by C/EBPα-p42. The HCNEs located on site C (chr6:150010787–150011203) of the *ULBP* gene sequence selectively mediated a strong response to C/EBPα-p42 (Figure 3(b)). The C/EBPa-binding region was approximately 4.7 kb upstream of the *ULBP1* transcription start site (TSS), 6.9 kb upstream of the *ULBP2* TSS, 8.4 kb upstream of the *ULBP5* TSS and 1.4 kb downstream of the *ULBP6* TSS. We further identified three sequences of C/EBPα BM DNA in site C (Figure 3(c)).

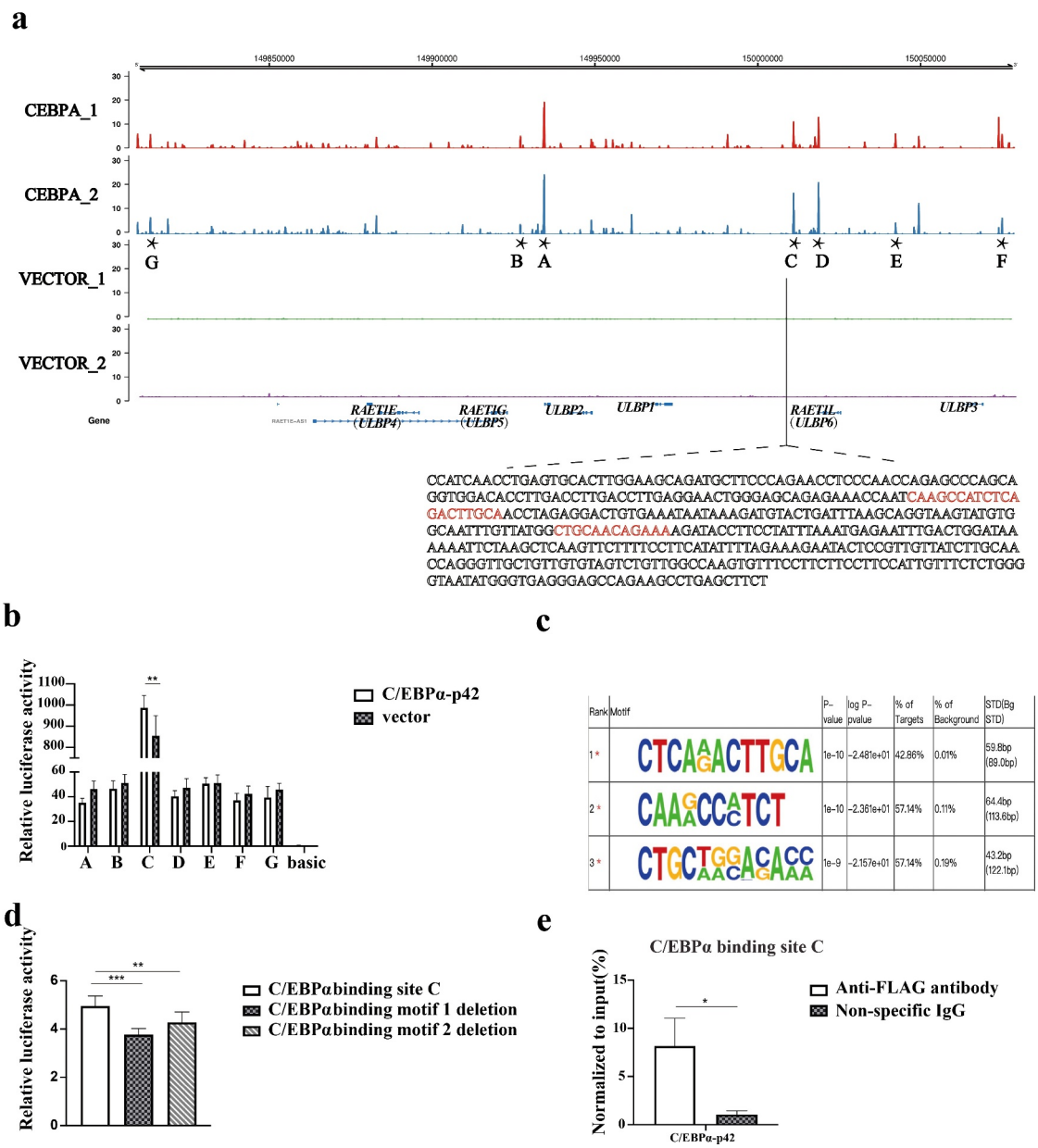

Figure 3. Wild-type C/EBPα activates *ULBP 2/5/6* transcription through direct binding to the enhancer regions of the *ULBPs* genes. (a). ChIP-seq revealed 7 C/EBPα-bound peaks in the *ULBP* genes in HEK293T cells. The 7 putative C/EBPα binding motifs (BMs) were located in highly conserved noncoding elements (HCNEs) of the *ULBP* genes assembly at chr6: 149934037–149934609 (A), chr6:149926955–149927208 (b), chr6:150010787–150011203 (c), chr6:150018361–150018857 (d), chr6:150042108–150042452 (e), chr6:150074859–150075176 (f), chr6:149813336–149813640 (g). Putative C/EBPα binding sites are shown as "*". **(B).** Seven HCNEs including the putative C/EBPα BM DNA fragment (100–200bp up- and down-stream relative to the respective sequence) on the *ULBPs* locus were cloned into luciferase reporter plasmids (pGL3 ULBPs). The luciferase activities normalized by Renilla luciferase activities were calculated for the promoter activity of the *ULBP* genes in HEK293T cells transfected with the C/EBPα-p42 expression construct and the control vector. **(C).** Identification of three sequences of C/EBPα BM DNA in site C. **(D).** The promoter activity of *ULBP* genes decreased significantly when the three C/EBPα BM DNA in site C in the *ULBP* genes were deleted (experiments used the psiCHECK2 plasmid). Because the NO. 1 sequence of C/EBPα BM DNA and the NO. 2 sequence of C/EBPα BM DNA are continuous, C/EBPα binding motif 1 deletion is simultaneously deleted the NO. 1 sequence and the NO. 2 sequence. **(E).** ChIP-qPCR confirmed that C/EBPα-p42 is an enhancer of the *ULBP* gene promoter.

To confirm the functional contribution of the C/EBPα binding site C to the *ULBP* promoter activity, we introduced the deletion of these three C/EBPα BM DNA in site C in the psiCHECK-*ULBP*-promoter reporter construct and measured the promoter activity mediated by C/EBPα-p42 using the luciferase activity assay. As shown in Figure 3(d), the promoter activity of the *ULBP* genes decreased significantly when the three C/EBPα BM sites in the *ULBP* genes were deleted, indicating that the activation of *ULBP* transcription by C/EBPα-p42 might occur through these consensus motifs.

Consistent with the ChIP-seq data, ChIP-qPCR confirmed C/EBPα-p42 as an enhancer of the *ULBP* gene promoter (Figure 3(e)).

CEBPA somatic mutants interfere with the expression of ULBP 2/5/6 in AML cells through the abrogated or reduced ability of binding to the ULBP gene enhancer sites

We hypothesized that *CEBPA* gene mutations might confer an immunosuppressive phenotype or immune evasion properties to AML cells. To address this question, we first chose the NB4 cell line, which has low endogenous C/EBPα-p42 expression and is resistant to NK-mediated cell lysis, as shown by our data described above (Figure 1(c)). The NB4 cell line was chosen to be transfected with the expression vectors of wild-type C/EBPα (pHIV7/SFFV-GFP-*CEBPA*-p42) and its three typical mutants, including the N-terminal truncated mutant (pHIV7/SFFV-GFP-*CEBPA*-p30), the C-terminal mutant (pHIV7/SFFV-GFP-*CEBPA*-C^m) and the germ-line mutant (pHIV7/SFFV-GFP-*CEBPA*-G^m), as described previously[16] (Figure 4(a)). We then examined the percentages of NK lysis by co-culture of the transfected NB4 cells with NK cells at an E:T ratio of 10:1. As shown in Figure 4(b), transfection with C/EBPα-p42 into NB4 cells significantly increased the sensitivity to NK-induced lysis to 41.5% compared with 5.5% in the cells transfected with empty vector as the control cohort ($P < .01$). NB4 cells transfected with the germ-line mutant (*CEBPA*-G^m) showed the same effect, in which the percentage of NK lysis was 20.3% in the transfected cohort vs 5.5% in the control cohort ($P < .001$). Furthermore, transfection of C/EBPα-p42 or *CEBPA*-G^m into NB4 cells induced significantly higher levels of expression of ULBP2/5/6 (Figure 4(c)). In contrast, the *CEBPA* somatic mutations, C/EBPα p30 and the C-terminal mutant (*CEBPA* -C^m), had no effect on induction of the sensitivity to NK-mediated cell lysis and expression of ULBP2/5/6 in the AML cells (Figure 4(b,c)).

To investigate whether the three mutants of C/EBPα directly impair the transcription of the *ULBP* genes, we first performed ChIP-qPCR in HEK293T cells expressing Flag-C/EBPα-p42, Flag-C/EBPα-p30, Flag- *CEBPA* -C^m and Flag- *CEBPA* -G^m. Primers specific to the HCNEs located on site C (chr6: 150010787–150011203) of the *ULBP* gene sequence were used for the PCR reaction. We observed that the binding activities of C/EBPα-p42, C/EBPα-p30 and *CEBPA*-G^m to C/EBPα BM in the *ULBP* genes increased dramatically, whereas the mutant *CEBPA* -C^m abrogated the binding to C/EBPα BM in the *ULBP* genes (Figure 4(d)). Next, to confirm whether the binding of C/EBPα-p30 to C/EBPα BM in the *ULBP* genes can directly activate *ULBP* promoter activity, we prepared a reporter plasmid in which the HCNEs located on site C (chr6: 150010787–150011203) of the human *ULBP* gene promoter were linked to the luciferase gene. Co-transfection of the C/EBPα-p42 expression construct increased the relative luciferase activity of the *ULBP* promoter. On the other hand, the expression of C/EBPα-p30 did not increase the *ULBP* promoter activity (Figure 4(e)).

Decitabine and LSD1 inhibition restore expression of ULBPs in AML cells with low expression of C/EBPα and induce their susceptibility to NK-mediated lysis in vitro and in vivo

The *CEBPA* transcripts are more susceptible to instability.[1] Studies reported transcriptional deregulation of *CEBPA* either by DNA methylation or by the recruitment of transcriptional repressor complexes to regulatory elements in the *CEBPA* locus.[18] Hypermethylation of the *CEBPA* promoter region

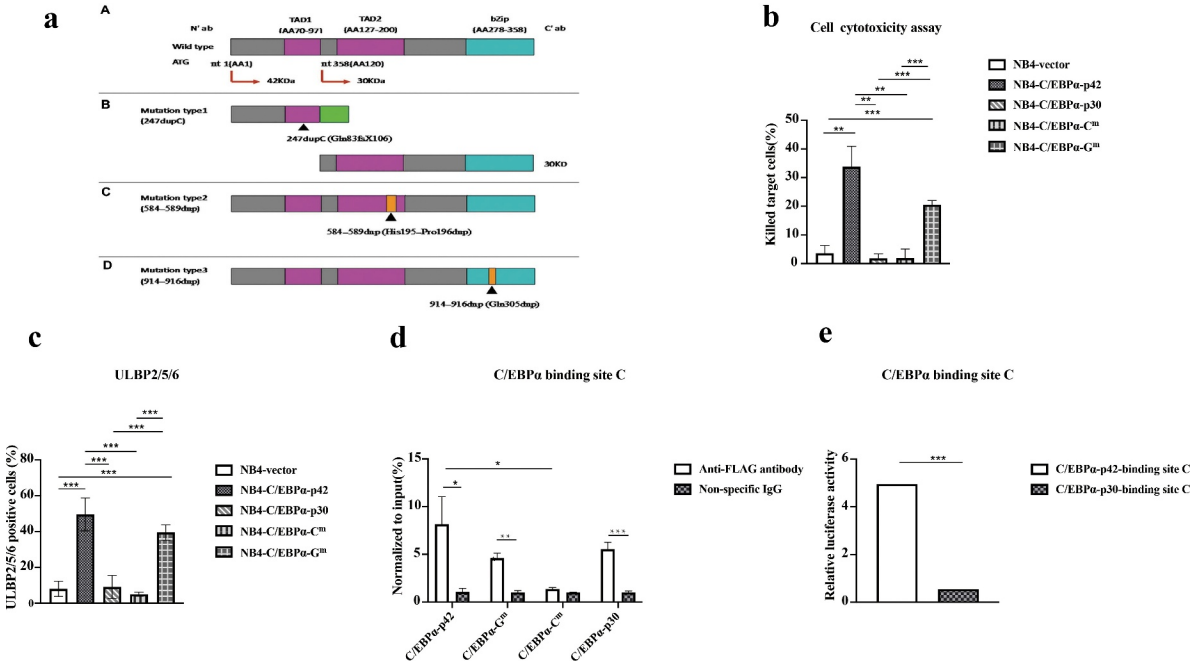

Figure 4. Effects and mechanisms of C/EBPα mutants on the expression of ULBP 2/5/6 in AML cells. (a). Schematic diagram of C/EBPα expression constructs and C/EBPα mutants. The germ-line mutant (584_589dup) (*CEBPA*-G^m), the C-terminal in-frame mutant (914_916dup) (*CEBPA*-C^m) and the N-terminal truncated mutation represented by the p30 isoform (C/EBPα-p30) were subcloned into Flag-tagged p3× FLAG-Myc-CMV and pHIV7/SFFV-GFP lentiviral plasmids. (b–c). NB4 cells were transfected with C/EBPα expression lentiviral plasmids of wild type and mutants. Flow cytometric analysis determined the percentage of NK lysis by co-culture of transfected NB4 cells with NK cells at an E:T ratio of 10:1 (b) and flow cytometric analysis detected the expression of ULBP2/5/6 in AML cells (c). (d). ChIP-qPCR analysis of HEK293T cells expressing Flag-C/EBPα-p42, Flag-C/EBPα-p30, Flag- *CEBPA* -C^m and Flag- *CEBPA* -G^m. Primers specific to the HCNEs located on site C (chr6:150010787–150011203) of the *ULBP* gene sequence were used for the PCR reaction. (e). Luciferase activity assay of the promoter activity of *ULBP* genes in HEK293T cells transfected with C/EBPα-p42 or C/EBPα-p30 expression constructs (experiments used the psiCHECK2 plasmid).

has been reported to result in down-regulation of *CEBPA* expression in AML and to be as a prognostic biomarker.[19–22] On the other hand, we proceeded on the basis of the reported results of the potential of hypomethylating agents (HMAs) to up-regulate NK-activating molecules, such as NKG2D ligands, on tumor cells through their epigenetic modulation, and thereby, sensitize tumors to NK-cell-mediated killing for different cancers including AML.[23] Azacitidine/ 5-azacytidine (Vidaza, AZA) and decitabine/ 5-aza-29- deoxycytidine (Dacogen, DAC) are two HMAs currently used for the treatment of AML and myelodysplastic syndromes. We first evaluated the impacts of AZA and DAC on the expression of ULBPs in the AML cell lines. NB4 cells, a low endogenous C/EBPα-p42 and ULBP2/5/6 expressing cell line, were cultured in the presence or absence of AZA at 0.05 to 1 μM or DAC at 0.05 to 5 μM for 48 h. We demonstrated that DAC reactivated C/EBPα expression (Figure 5(a) and Supplementary Figure S2) and up-regulated the expression of ULBPs in the NB4 cells (Figure 5(b)). However, AZA had no significant effect on induction of the expression of ULBPs in the NB4 cells (Supplementary Figure S3).

To further explore novel promising drugs that could restore the expression of ULBPs in AML cells and increase their sensitivity to NK-mediated lysis, we screened 70 small molecular drugs (Supplementary Table 3), which were selected from a library containing 932 drugs targeting epigenetic regulators, to treat NB4 cells for 48 h. Flow cytometry analysis identified the efficacies for up-regulation of ULBP expression by the candidate drugs. Data shown as rate of positive cells and MFI in Figure 5(c-e) and Supplementary Figure S4, three of the drugs were identified as having a significant ability to up-regulate the expression levels of ULBPs in NB4 cells. Tranylcypromine (2-PCPA) hydrochloride, which is a lysine-specific demethylase 1 (LSD1) inhibitor; clevudine, which is a DNA/RNA synthesis inhibitor, and methyl cinnamate, which is a tyrosinase inhibitor, all could significantly up-regulate ULBP expression in the NB4 cells. The levels of expression of the ULBPs increased as did their concentrations. 2-PCPA was the most effective drug for up-regulating ULBP expression. After treatment with 1 mM 2-PCPA for 2 days, the levels of expression of ULBP1, ULBP2/5/6 and ULBP3 in NB4 cells increased from 2.61 ± 0.61% to 23.7 ± 2.11% ($P < .001$), 2 ± 0.58% to 40.43 ± 6.50% ($P < .001$) and 3.57 ± 0.43% to 18.33 ± 0.59% ($P < .001$), respectively. However, DAC, 2-PCPA, clevudine and methyl cinnamate all have no significant effect on MIC A/B expression in NB4 cells (Supplementary Figure S5)

We further revealed that LSD1 inhibition (2-PCPA) exhibited robust induction of the expression of *CEBPA* in AML cells measured by RT-qPCR and Western blotting (Figure 6(a) and Supplementary Figure S6). In *vitro* experiment, before being co-cultured with NK cells at an E:T ratio of 10:1, treatment with 1 mM 2-PCPA for 24 h significantly increased the sensitivity of NB4 cells to NK-induced lysis to 50.65 ± 0.31% compared with 43.9 ± 1.56% in the control cells ($P < .01$) (Figure 6(b)). Finally, we investigated the combinatorial effects of LSD1 inhibition and NK cell therapy against AML *in vivo*. We established a humanized AML animal model with NCG mice by injection of 2×10^5 NB4 cells labeled with luciferase through the tail vein. The mice in the treatment group received a single dose of 1.2×10^6 primary NK cells, or intraperitoneal injection of a single dose of 15 mg/kg 2-PCPA, or

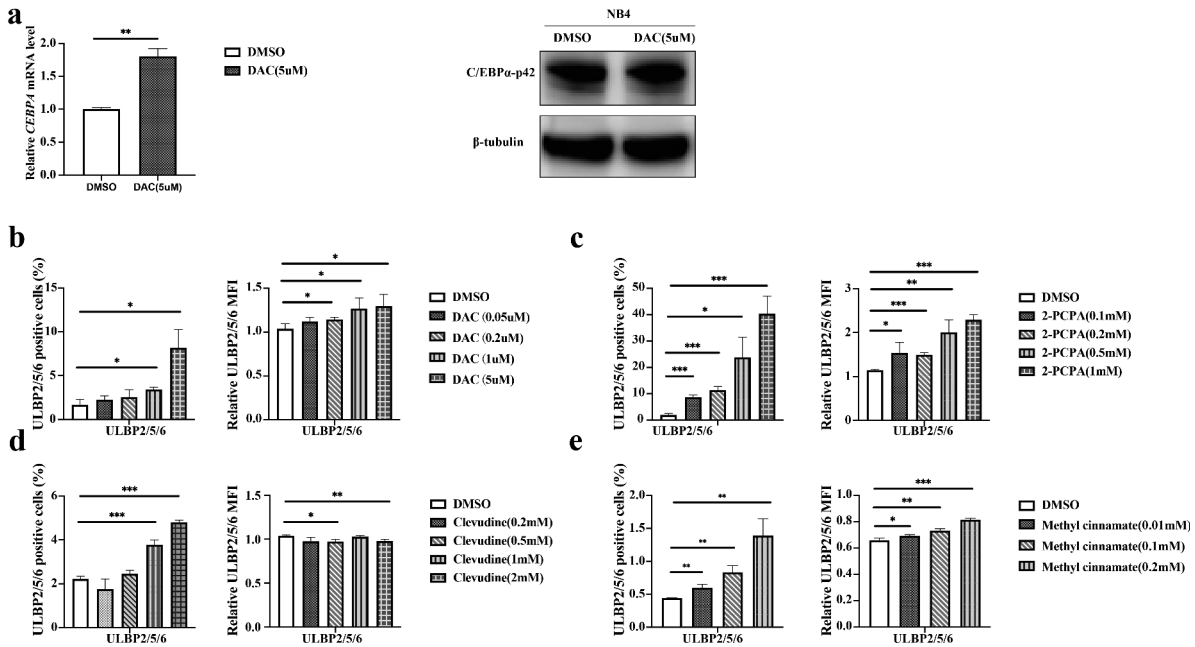

Figure 5. Decitabine and small molecular compounds restore expression of ULBPs in AML cells with low expression of C/EBPα and induce their susceptibility to NK-mediated lysis in *vitro*. (a). The impact of decitabine (DAC) on the expression of C/EBPα in AML cell lines. NB4 cells, a low endogenous C/EBPα-p42 and ULBP2/5/6 expressing cell line, were cultured in the presence or absence of DAC at 0.05 to 5 μM for 48 h. RT-qPCR (Figure A left) and Western blotting analysis (Figure A right) confirmed that DAC reactivated C/EBPα expression in NB4 cells after treatment with 5 μM DAC for 48 h. (b). DAC up-regulated the expression of ULBP2/5/6 on NB4 cells. Data were presented as rate of positive cells and MFI. (c–e). Three kinds of small molecular drugs were identified to have a significant ability to up-regulate the expression levels of ULBP2/5/6 on NB4 cells after treatment for 48 h: tranylcypromine (2-PCPA) hydrochloride (c), clevudine (d) and methyl cinnamate (e). Data were presented as rate of positive cells and MFI.

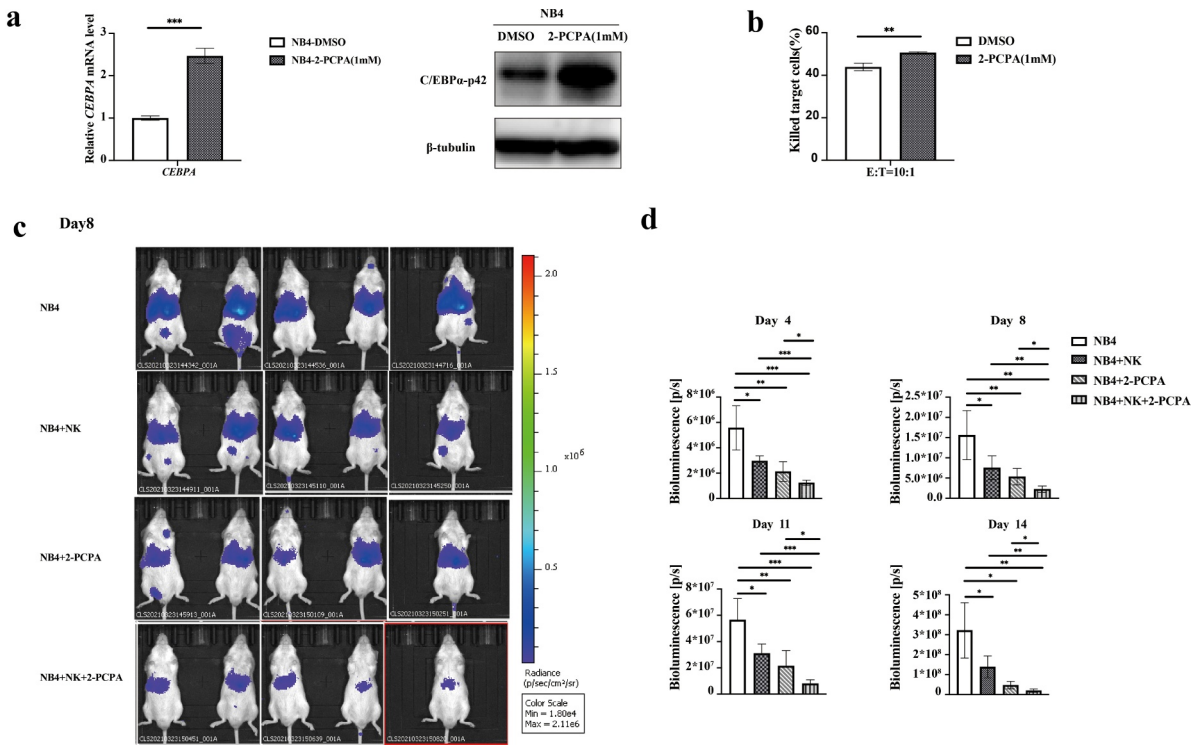

Figure 6. LSD1 inhibition restores the expression of ULBPs in AML cells with low expression of C/EBPα and induce their susceptibility to NK-mediated lysis in *vivo*. (a). Quantitative real-time PCR (left) and Western blotting analysis (right) showed that LSD1 inhibition (2-PCPA) induced the expression of C/EBPα in NB4 cells after treatment with 1 mM 2-PCPA for 48 h. (b). In *vitro* experiment, before being co-cultured with NK cells at an E:T ratio of 10:1, treatment with 1 mM 2-PCPA for 24 h significantly increased the sensitivity of NB4 cells to NK-induced lysis to 50.65 ± 0.31% compared with 43.9 ± 1.56% in the control cells ($P < .01$). (c). Bioluminescence imaging revealed the tumor load in mice in the control, after treatment with LSD1 inhibition 2-PCPA, human NK cells, human NK cells in combination with LSD1 inhibition 2-PCPA for 8 days. (d). Quantification of bioluminescence showed that lower tumor load in mice after treatment with human NK cells for 4 days, 8 days, 11 days and 14 days, compared with control animals. This reduction in tumor progression was most significant after treatment with human NK cells in combination with LSD1 inhibition for 4 days to 2 weeks, compared with LSD1 inhibition treatment alone or human NK cell treatment alone. Each experimental group included 5 mice.

combination treatment of NK cells and 2-PCPA, respectively. The mice in the control group were injected with PBS. Bioluminescence imaging revealed a lower tumor load in mice after treatment with human NK cells for 4 days, 8 days, 11 days and 14 days, compared with control animals. More importantly, this reduction in tumor progression was most significant after treatment with human NK cells in combination with LSD1 inhibition for 4 days to 2 weeks, compared with LSD1 inhibition treatment alone or human NK cell treatment alone ($P < .01$) (Figure 6(c,d)). We further repeated the Xenograft model experiment using a different donor to avoid inter-donor variability. Quantification of bioluminescence also showed that lower tumor load in mice after treatment with human NK cells in combination with LSD1 inhibition for 4 days to 2 weeks, compared with human NK cell treatment alone (Supplementary Figure S7).

Discussion

To the best of our knowledge, this is the first study to demonstrate a role for C/EBPα in the transcriptional control of *ULBP* gene expression, and to confirm the importance of this transcription factor in the regulation of sensitivity to NK-mediated lysis in AML cells. Furthermore, we demonstrated for the first time an inverse relationship between *CEBPA* somatic mutations and expression of *ULBPs*.

The pathways and signals known to regulate NKG2D ligands involve transcription, RNA stabilization, translation, protein stabilization, cell surface egress and excretion/shedding of ligands from cells.[24] It is generally assumed that most regulation of NKG2D ligands occurs at the transcriptional stage. The *ULBP* gene family consists of 10 loci, with six of them encoding functional transcripts (*ULBP1–6*). Some studies have reported that transcriptional factors involved in leukemogenesis play a critical role in regulating the expression of *ULBPs*. Unni *et al* have provided evidence that expression of NKG2D ligands is induced at an early, distinct stage of tumorigenesis upon acquisition of genetic lesions unique to cancer cells and that c-Myc is involved in their regulation.[25] Nanbakhsh *et al* further revealed a direct correlation between c-Myc activation and *ULBP1/3* expression.[11] Activated p53 can enhance the transcription of certain NKG2D ligands, such as ULBP1 and 2.[10,26] Furthermore, p53 was indispensable for the induction of NKG2D ligands by DNA-damaging agents.[27] In addition, RAS activation is also associated with the induction of the NKG2D ligands ULBP1–3 in human cells.[28] In the present study, we provided further data on the correlation between those two important genes, NKG2D ligands, *ULBP2/5/6* and *CEBPA*, which are involved in the immune response and leukemogenesis, respectively. ChIP studies revealed that wild-type C/EBPα (C/EBPα-p42) can regulate ULBP2/5/6 expression by directly

binding the enhancer of *ULBP2/5/6* genes through *CEBPA* BMs. More intriguingly, we provided evidence to demonstrate that the AML-associated C/EBPα mutant p30 and C/EBPα C-terminal mutant lose this ability.

It is reasonable to conclude that the C/EBPα C-terminal mutant (C/EBPα-C^m) had an attenuated ability to bind to the *ULBP* genes because of in-frame mutations in the C-terminal region that alter the basic leucine zipper domain, leading to the loss of DNA binding. On the other hand, ChIP-qPCR detection showed that C/EBPα-p30 retained the ability to bind to the *ULBP* genes. However, the expression of C/EBPα-p30 did not increase the *ULBP* promoter activity according to luciferase reporter analysis. In line with these results, the enforced expression of C/EBPα-p30 in the AML cell line had no effect on induction of the expression of ULBP2/5/6 or increasing the sensitivity of AML cells to NK-mediated cell lysis. The C/EBPα-p30 isoform represents the most prevalent type of *CEBPA* mutation in AML, and the mechanistic basis of p30-induced leukemogenesis is incompletely understood. N-terminal deletions strongly reduce transcriptional activation by C/EBPα. Yet, certain properties that are exhibited by the p42 isoform are still maintained by the p30 variant. It is proposed that the C/EBPα-p42 and p30 isoforms might have variable affinities for consensus C/EBP binding sites in the genome.[29] Elizabeth Heyes *et al* revealed a comprehensive dataset of p30-dependent effects on epigenetic regulation and gene expression, and identified RNA-binding protein (RBP) MUSASHI-2 (MSI2) as an effector of the C/EBPα-p30 oncoprotein.[30] Furthermore, C/EBPα-p30 is able to actively change the gene expression pattern of leukemia cells through its ability to bind to chromatin and consequently recruit chromatin modulators, such as histone methyltransferases including Wdr5, a key component of SET/MLL histone-methyltransferase complexes,[31] and MLL1 histone-methyltransferase complex.[32]

Finally, given the impairment of NK-cell cytotoxicity in most hematologic malignancies, the restoration of normal NK function or adoptive NK cell therapy remains an attractive option for treating leukemia patients. Numerous small molecules, some of which already are used in clinical practice, have been shown to modulate the activity of NK cells and to enhance the expression of NKG2D-Ls on tumor cells, such as the inhibitor of histone deacetylases (HDACi) valproate (VPA), the proteasomal inhibitor bortezomib and the DNA methyl transferase inhibitor (DNMTi) 5-aza-2′-deoxycytidine (decitabine, DAC).[33] We demonstrated that DAC, but not AZA, up-regulated the expression of ULBPs in AML cells with low endogenous C/EBPα-p42 expression, which is consistent with the published report that only DAC, not AZA, up-regulated NKG2D- and DNAM-1-activating ligands on AML cells *in-vivo*.[34] Interestingly, we also first revealed that the LSD1 inhibitor, tranylcypromine (2-PCPA), can restore the expression of ULBPs via induction of *CEBPA* expression on AML cells and induce their susceptibility to NK-mediated lysis *in vitro* and *in vivo*. LSD1, an H3K4/9me eraser, could regulate gene expression genome wide during carcinogenesis. Targeting LSD1 is becoming an emerging option for the treatment of cancers. Numerous LSD1 inhibitors have been reported to date, and some of them such as TCP, ORY-1001, GSK-2879552, IMG-7289, INCB059872, CC-90011 and ORY-2001 are currently undergoing clinical assessment for cancer therapy, particularly for small cell lung cancer (SCLC) and AML.[35] Recently, a phase I/II pilot trial showed that treatment with LSD1 inhibition by tranylcypromine combined with all-trans retinoic acid (ATRA) induced the differentiation of AML blasts and led to a clinical response in heavily pretreated patients with refractory/relapsed AML with acceptable toxicity.[36] It is reasonable to surmise that the future of immunotherapy in AML lies in the rational combination of complementary immunotherapeutic strategies with chemotherapeutics or other oncogenic pathway inhibitors.

Collectively, our results identify a critical link between C/EBPα and its mutants and NKG2D ligand expression and sensitivity to NK-mediated lysis in AML cells, and show that restoration of NKG2D ligand expression could represent a novel therapeutic strategy for patients with *CEBPA*-mutated AML.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

HW Xiao and LM Wang designed the research study; M Liu, LM Wang, MB Du, J Yu, MW Wang, HM Li, JQ Zheng and QSh Huang performed the experiments; WJ Pan, XJ Zhao and HW Xiao provided study materials or patients. M Liu, HW Xiao and LM Wang analyzed the data; HW Xiao wrote the paper, LM Wang revised the paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study has been approved by the Ethics Committee and written informed consent was obtained from all participants.

Consent for publication

The authors declare that they consent for publication.

References

1. Avellino R, Delwel R. Expression and regulation of C/EBPalpha in normal myelopoiesis and in malignant transformation. Blood. 2017;129(15):2083–2091. doi:10.1182/blood-2016-09-687822.
2. Koschmieder S, Halmos B, Levantini E, Tenen DG. Dysregulation of the C/EBPalpha differentiation pathway in human cancer. J Clin Oncol. 2009;27(4):619–628. e-pub ahead of print 2008/12/17; doi: 10.1200/JCO.2008.17.9812JCO.2008.17.9812 [pii].
3. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001;27(3):263–270. doi:10.1038/85820.
4. Pabst T, Eyholzer M, Haefliger S, Schardt J, Mueller BU. Somatic CEBPA mutations are a frequent second event in families with germline CEBPA mutations and familial acute myeloid leukemia. J Clin Oncol. 2008;26(31):5088–5093. e-pub ahead of print 2008/09/05; doi: 10.1200/JCO.2008.16.5563 [pii].
5. Taskesen E, Bullinger L, Corbacioglu A, Sanders MA, Erpelinck CA, Wouters BJ, van der Poel-van de Luytgaarde SC, Damm F, Krauter J, Ganser A, *et al*. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. Blood. 2011;117(8):2469–2475. doi:10.1182/blood-2010-09-307280.
6. Pathak A, Seipel K, Pemov A, Dewan R, Brown C, Ravichandran S, Luke BT, Malasky M, Suman S, Yeager M, *et al*. Whole exome sequencing reveals a C-terminal germline variant in CEBPA-associated acute myeloid leukemia: 45-year follow up of a large family. Haematologica. 2016;101(7):846–852. doi:10.3324/haematol.2015.130799.
7. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285(5428):727–729. doi:10.1126/science.285.5428.727.
8. Nowbakht P, Ionescu MC, Rohner A, Kalberer CP, Rossy E, Mori L, Cosman D, De Libero G, Wodnar-Filipowicz A. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood. 2005;105(9):3615–3622. doi:10.1182/blood-2004-07-2585.
9. Paczulla AM, Rothfelder K, Raffel S, Konantz M, Steinbacher J, Wang H, Tandler C, Mbarga M, Schaefer T, Falcone M, *et al*. Absence of NKG2D ligands defines leukaemia stem cells and mediates their immune evasion. Nature. 2019;572(7768):254–259. doi:10.1038/s41586-019-1410-1.
10. Textor S, Fiegler N, Arnold A, Porgador A, Hofmann TG, Cerwenka A. Human NK cells are alerted to induction of p53 in cancer cells by upregulation of the NKG2D ligands ULBP1 and ULBP2. Cancer Res. 2011;71(18):5998–6009. doi:10.1158/0008-5472.CAN-10-3211.
11. Nanbakhsh A, Pochon C, Mallavialle A, Amsellem S, Bourhis JH, Chouaib S. c-Myc regulates expression of NKG2D ligands ULBP1/2/3 in AML and modulates their susceptibility to NK-mediated lysis. Blood. 2014;123(23):3585–3595. doi:10.1182/blood-2013-11-536219.
12. Bedel R, Thiery-Vuillemin A, Grandclement C, Balland J, Remy-Martin JP, Kantelip B, Pallandre J-R, Pivot X, Ferrand C, Tiberghien P, *et al*. Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells. Cancer Res. 2011;71(5):1615–1626. doi:10.1158/0008-5472.CAN-09-4540.
13. Pulikkan JA, Tenen DG, Behre G. C/EBPalpha deregulation as a paradigm for leukemogenesis. Leukemia. 2017;31(11):2279–2285. doi:10.1038/leu.2017.229.
14. Garcia-Iglesias T, Del Toro-Arreola A, Albarran-Somoza B, Del Toro-Arreola S, Sanchez-Hernandez PE, Ramirez-Duenas MG, Balderas-Peña LMA, Bravo-Cuellar A, Ortiz-Lazareno PC, Daneri-Navarro A, *et al*. Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions. BMC Cancer. 2009;9(1):186. doi:10.1186/1471-2407-9-186.
15. Raneros AB, Minguela A, Rodriguez RM, Colado E, Bernal T, Anguita E, Mogorron AV, Gil AC, Vidal-Castiñeira JR, Márquez-Kisinousky L, *et al*. Increasing TIMP3 expression by hypomethylating agents diminishes soluble MICA, MICB and ULBP2 shedding in acute myeloid leukemia, facilitating NK cell-mediated immune recognition. Oncotarget. 2017;8(19):31959–31976. doi:10.18632/oncotarget.16657.
16. Xiao H, Shi J, Luo Y, Tan Y, He J, Xie W, Zhang L, Wang Y, Liu L, Wu K, *et al*. First report of multiple CEBPA mutations contributing to donor origin of leukemia relapse after allogeneic hematopoietic stem cell transplantation. Blood. 2011;117(19):5257–5260. doi:10.1182/blood-2010-12-326322.
17. Wang L, Xiao H, Zhang X, Liao W, Fu S, Huang H. Restoration of CCAAT enhancer binding protein alpha P42 induces myeloid differentiation and overcomes all-trans retinoic acid resistance in human acute promyelocytic leukemia NB4-R1 cells. Int J Oncol. 2015;47(5):1685–1695. doi:10.3892/ijo.2015.3163.
18. Figueroa ME, Wouters BJ, Skrabanek L, Glass J, Li Y, Erpelinck-Verschueren CA, Langerak AW, Löwenberg B, Fazzari M, Greally JM, *et al*. Genome-wide epigenetic analysis delineates a biologically distinct immature acute leukemia with myeloid/T-lymphoid features. Blood. 2009;113(12):2795–2804. doi:10.1182/blood-2008-08-172387.
19. Lin TC, Hou HA, Chou WC, Ou DL, Yu SL, Tien HF, Lin L-I. CEBPA methylation as a prognostic biomarker in patients with de novo acute myeloid leukemia. Leukemia. 2011;25(1):32–40. doi:10.1038/leu.2010.222.
20. Lu Y, Chen W, Chen W, Stein A, Weiss LM, Huang Q. C/EBPA gene mutation and C/EBPA promoter hypermethylation in acute myeloid leukemia with normal cytogenetics. Am J Hematol. 2010;85(6):426–430. doi:10.1002/ajh.21706.
21. Jost E, Wilop S, Herman JG, Osieka R, Galm O. Aberrant DNA methylation of the transcription factor C/EBPalpha in acute myelogenous leukemia. Leuk Res. 2009;33(3):443–449. doi:10.1016/j.leukres.2008.07.027.
22. Chim CS, Wong AS, Kwong YL. Infrequent hypermethylation of CEBPA promotor in acute myeloid leukaemia. Br J Haematol. 2002;119(4):988–990. doi:10.1046/j.1365-2141.2002.03952.x.
23. Baragano Raneros A, Martin-Palanco V, Fernandez AF, Rodriguez RM, Fraga MF, Lopez-Larrea C, Suarez-Alvarez B, *et al*. Methylation of NKG2D ligands contributes to immune system evasion in acute myeloid leukemia. Genes Immun. 2015;16(1):71–82. doi:10.1038/gene.2014.58.
24. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. Annu Rev Immunol. 2013;31:413–441. doi:10.1146/annurev-immunol-032712-095951.
25. Unni AM, Bondar T, Medzhitov R. Intrinsic sensor of oncogenic transformation induces a signal for innate immunosurveillance. Proc Natl Acad Sci U S A. 2008;105(5):1686–1691. doi:10.1073/pnas.0701675105.
26. Li H, Lakshmikanth T, Garofalo C, Enge M, Spinnler C, Anichini A, Szekely L, Kärre K, Carbone E, Selivanova G, *et al*. Pharmacological activation of p53 triggers anticancer innate immune response through induction of ULBP2. Cell Cycle. 2011;10(19):3346–3358. doi:10.4161/cc.10.19.17630.
27. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature. 2005;436(7054):1186–1190. doi:10.1038/nature03884.
28. Liu XV, Ho SS, Tan JJ, Kamran N, Gasser S. Ras activation induces expression of Raet1 family NK receptor ligands. J Immunol. 2012;189(4):1826–1834. doi:10.4049/jimmunol.1200965.

29. Cleaves R, Wang QF, Friedman AD. C/EBPalphap30, a myeloid leukemia oncoprotein, limits G-CSF receptor expression but not terminal granulopoiesis via site-selective inhibition of C/EBP DNA binding. Oncogene. 2004;23(3):716–725. doi:10.1038/sj.onc.1207172.
30. Heyes E, Schmidt L, Manhart G, Eder T, Proietti L, Grebien F. Identification of gene targets of mutant C/EBPalpha reveals a critical role for MSI2 in CEBPA-mutated AML. Leukemia. 2021;35(9):2526–2538. doi:10.1038/s41375-021-01169-6.
31. Grebien F, Vedadi M, Getlik M, Giambruno R, Grover A, Avellino R, Skucha A, Vittori S, Kuznetsova E, Smil D, *et al*. Pharmacological targeting of the Wdr5-MLL interaction in C/EBPalpha N-terminal leukemia. Nat Chem Biol. 2015;11(8):571–578. doi:10.1038/nchembio.1859.
32. Schmidt L, Heyes E, Scheiblecker L, Eder T, Volpe G, Frampton J, Nerlov C, Valent P, Grembecka J, Grebien F, *et al*. CEBPA-mutated leukemia is sensitive to genetic and pharmacological targeting of the MLL1 complex. Leukemia. 2019;33(7):1608–1619. doi:10.1038/s41375-019-0382-3.
33. Lazarova M, Steinle A. The NKG2D axis: an emerging target in cancer immunotherapy. Expert Opin Ther Targets. 2019;23(4):281–294. doi:10.1080/14728222.2019.1580693.
34. Cany J, Roeven MWH, Hoogstad-van Evert JS, Hobo W, Maas F, Franco Fernandez R, Blijlevens NMA, van der Velden WJ, Huls G, Jansen JH, *et al*. Decitabine enhances targeting of AML cells by CD34(+) progenitor-derived NK cells in NOD/SCID/IL2Rg(null) mice. Blood. 2018;131(2):202–214. doi:10.1182/blood-2017-06-790204.
35. Fang Y, Liao G, Yu B. LSD1/KDM1A inhibitors in clinical trials: advances and prospects. J Hematol Oncol. 2019;12(1):129. doi:10.1186/s13045-019-0811-9.
36. Wass M, Gollner S, Besenbeck B, Schlenk RF, Mundmann P, Gothert JR, Noppeney R, Schliemann C, Mikesch J-H, Lenz G, *et al*. A proof of concept phase I/II pilot trial of LSD1 inhibition by tranylcypromine combined with ATRA in refractory/relapsed AML patients not eligible for intensive therapy. Leukemia. 2021;35(3):701–711. doi:10.1038/s41375-020-0892-z.