Restoration of CCAAT enhancer binding protein α P42 induces myeloid differentiation and overcomes all-*trans* retinoic acid resistance in human acute promyelocytic leukemia NB4-R1 cells

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Abstract. All-*trans* retinoic acid (ATRA) is one of the first line agents in differentiation therapy for acute promyelocytic leukemia (APL). However, drug resistance is a major problem influencing the efficacy of ATRA. Identification of mechanisms of ATRA resistance are urgently needed. In the present study, we found that expression of C/EBPα, an important transcription factor for myeloid differentiation, was significantly suppressed in ATRA resistant APL cell line NB4-R1 compared with ATRA sensitive NB4 cells. Moreover, two forms of C/EBPα were unequally suppressed in NB4-R1 cells. Suppression of the full-length form P42 was more pronounced than the truncated form P30. Inhibition of PI3K/Akt/mTOR pathway was also observed in NB4-R1 cells. Moreover, C/EBPα expression was reduced by PI3K inhibitor LY294002 and mTOR inhibitor RAD001 in NB4 cells, suggesting that inactivation of the PI3K/Akt/mTOR pathway was responsible for C/EBPα suppression in APL cells. We restored C/EBPα P42 and P30 by lentivirus vectors in NB4-R1 cells, respectively, and found C/EBPα P42, but not P30, could increase CD11b, CD14, G-CSFR and GM-CSFR expression, which indicated the occurrence of myeloid differentiation. Further upregulating of CD11b expression and differential morphological changes were found in NB4-R1 cells with restored C/EBPα P42 after ATRA treatment. However, CD11b expression and differential morphological changes could not be induced by ATRA in NB4-R1 cells infected with P30 expressing or control vector. Thus, we inferred that ATRA sensitivity of NB4-R1 cells was enhanced by restoration of C/EBPα P42. In addition, we used histone deacetylase inhibitor trichostatin (TSA) to restore C/EBPα expression in NB4-R1 cells. Similar enhancement of myeloid differentiation and cell growth arrest were detected. Together, the present study demonstrated that suppression of C/EBPα P42 induced by PI3K/Akt/mTOR inhibition impaired the differentiation and ATRA sensitivity of APL cells. Restoring C/EBPα P42 is an attractive approach for differentiation therapy in ATRA resistant APL.

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Abbreviations: C/EBPα, CCAAT enhancer binding protein α; ATRA, all-*trans* retinoic acid; APL, acute promyelocytic leukemia; HDACi, histone deacetylase inhibitor; TSA, trichostatin

Key words: CCAAT enhancer binding protein α, acute promyelocytic leukemia, all-*trans* retinoic acid, differentiation therapy, drug resistance, histone deacetylase inhibitor

Introduction

Acute promyelocytic leukemia (APL) is a specific type of acute myeloid leukemia (AML). Most (98%) of APL patients harbor the t(15;17) translocation, that leads to the expression of the fusion protein promyelocytic leukemia-retinoic acid receptor α (PML-RARα) (1-3). PML-RARα recruits corepressor complexes N-CoR/SMRT and polycomb repressive complex 1/2 to promoters of a series of target genes and microRNA, resulting in their transcriptional alteration (4-7). All-*trans* retinoic acid (ATRA) is one of the first line drugs in the induction therapy of APL. Since the introduction of ATRA more than 80% of APL patients achieve complete remission (CR) and most of them obtained satisfactory health-related quality-of-life (8,9). However, there is still a section of APL patients who do not respond well to ATRA treatment, with a resulting shorter survival. Drug resistance of ATRA is a serious obstacle for its clinical efficiency.

Several mechanisms of ATRA resistance in APL cells have been proposed (10). PLZF-RARα and STAT5b-RARα fusion proteins (4,11), increased catabolism of ATRA and the presence of the cytoplasmic retinoic acid binding protein (CRABP) are considered as reasons for ATRA resistance (12-14). However, only genetic mutations in the ligand binding domain (LBD) of RARα have been

confirmed as a mechanism of ATRA resistance. In the study by Côté *et al* (15), ATRA binding affinity of Cos-1 cells (with mutated PML-RARα) was lower than that of cell lines without PML-RARα mutations (NB4-R1, R2, R4 and RA) because of structural changes in their LBD domains. Gallagher *et al* (16) reported that 18 of 45 (40%) of relapsed APL patients, expressed the PML-RARα LBD mutation. However, mechanisms of ATRA resistance of APL cells without the PML-RARα mutations remain unknown.

Effective treatment of ATRA resistant APL is a serious clinical challenge. Although As_2O_3 was reported to rescue most relapsed/refractory patients treated with ATRA/chemotherapy, its severe side-effects limit its long-term use (17). Some natural compounds, pharmaceuticals and siRNA have also been tested to transcriptionally enhance activation of PML-RARα target genes (18-21). Novel effective approaches to enhance ATRA sensitivity in ATRA resistant APL cells are still urgently needed.

Transcription factor CCAAT enhancer binding protein α (C/EBPα) plays an important role in early hematopoiesis. C/EBPα activates myeloid development of multiple potential progenitor cells and granulocyte-monocyte progenitors (GMP), as adult mice with a conditional knockout C/EBPα encoding gene-CEBPA are devoid of GMPs and consecutive granulocytes (22,23). Myeloid differentiation inducing effect of C/EBPα is very forceful, as enforced C/EBPα expression in B-cell acute lymphoblastic leukemia cells reprogrammed these cells into macrophages (24). CEBPA mutations are common in AML patients with normal karyotype while its transcriptional suppression is often observed in AML patients with fusion genes (25).

Besides the 42-kDa full-length protein (P42), C/EBPα protein has a 30-kDa truncated protein form (P30) which was translated from the same mRNA as P42. P30 is initiated at an in-frame AUG codon downstream of CEBPA mRNA, and thus lacks the first transactivation domains (TAD) at the N-terminus (26,27). Dominant negative C/EBPα P30 isoform loses ability to regulate many differentiation associated and antitumor genes but preserved growth arresting ability in AML cells (25,28). Moreover, P30 also has target genes distinct from P42, such as PIN1, microRNA-181a and long non-coding RNA UCA1 encoding genes (29-31). However, expression of C/EBPα P42 and P30 and their roles in ATRA resistant APL cells remain unknown. The present study shows that C/EBPα P42 and P30 were suppressed to different extent in ATRA resistant NB4-R1 cells. Restoring C/EBPα P42, but not P30, induced myeloid differentiation of NB4-R1 cells and enhanced their sensitivity to ATRA.

Materials and methods

Reagents. All-*trans* retinoic acid (ATRA) and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAD001 (everolimus) was a kind gift from Novartis (Basel, Switzerland).

Cells and cell culture. ATRA resistant APL cell line NB4-R1 was a kind gift from Dr J. Zhu (Shanghai Jiao Tong University School of Medicine, Shanghai, China). NB4 cells and 293T cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). NB4 and NB4-R1 were maintained in RPMI-1640 medium (Corning, Corning, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). The 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Corning) with 10% FBS. All cells were cultured in a humidified atmosphere of 95% air/5% CO_2 at 37˚C, and maintained at a density of $<5x10^5$ cells/ml.

Preparation of peripheral blood mononuclear cells (PBMC). Healthy volunteers with informed consent donated their blood cells for the study. The procedures received an official approval from the ethics committee of First Affiliated Hospital of Zhejiang University. Venous blood from each of the healthy volunteer was withdrawn into blood collecting tubes with sodium citrate, and diluted twice with phosphate-buffered saline (PBS). The diluted blood was slowly layered onto Lymphoprep™ and centrifuged at 800 x g for 20 min at room temperature (slow acceleration, no braking). Cells from the interphase (PBMC) were collected and washed with PBS by centrifugation at 300 x g for 10 min at room temperature.

Plasmids and lentivirus infection. The full-length CEBPA coding sequence (CEBPA, 1077 bp, NM_004364.2) and P30 coding sequence were subcloned into Flag-tagged pLenti6.3/V5-DEST plasmids (Invitrogen, Waltham, MA, USA). Lentivirus was produced by co-transfecting the packaging plasmids (PSPAX2 and PMD2.G) with lentivirus vectors into 293T cells, using the Attractene transfection reagent (Qiagen, Valencia, CA, USA). Supernatants containing lentivirus were harvested 72 h after transfection, filtered by a 4.5 μm filter and purified using 10% PEG8000 (Sigma-Aldrich).

Lentivirus preparations were diluted in 1 ml complete medium containing 8 mg/ml polybrene (Sigma-Aldrich), and added to the cells for 12 h of incubation at 37˚C, followed by incubation in 1 ml of fresh complete medium. Positive clones were selected by 10 μg/ml blasticidin (Invitrogen) at day 5 after infection.

Methyl thiazolyl tetrazolium (MTT) assay. Proliferation of NB4 and NB4-R1 cells was assessed using the MTT assay. Briefly, reconstituted MTT was added to medium of treated cells and incubated for 4 h. Then, formazan was dissolved by DMSO solvent. Absorbance at 570 nm was recorded using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The following equation was used: Proliferation inhibition rate (%) = (control group OD_{570} - experimental group OD_{570})/(control group OD_{570} - medium control OD_{570}) x 100%).

Giemsa staining. The cell morphology was determined with Giemsa staining. Briefly, cells were centrifuged onto slides at 200 x g for 5 min, fixed with methanol for 10 min, and stained with Giemsa stain (Sigma-Aldrich) for 5 min. Slides were washed with distilled water and viewed by a microscope (Nikon, Tokyo, Japan) at x400 magnification.

Flow cytometric (FCM) analysis. Cells were harvested and washed with staining buffer (0.5% bovine serum albumin in PBS) by centrifuge at 300 x g for 10 min. Then, cells were

resuspended with 100 μl staining buffer and incubated with 5 μl fluorophore-conjugated antibodies at 4˚C in the dark for 30 min. Mouse anti-human FITC-CD11b, FITC-CD14, APC-CD114, or FITC-CD116 antibodies (BioLegend, San Diego, CA, USA) were used in the experiments. Then cells were washed twice with staining buffer. Fluorescent intensities were determined using flow cytometry (Beckman Coulter, Inc., Miami, FL, USA) and isotype antibodies were used to assess non-specific staining.

RNA extraction and polymerase chain reaction (PCR). Total RNA were extracted from NB4-R1 cells using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA) and their concentration were detected by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed to cDNA using Takara PrimeScript RT reagent kit (Takara, Tokyo Japan), according to the manufacturer's instructions.

PCR analysis was performed using a Takara Taq Recombinant Taq DNA polymerase kit (Takara). The *SPI1* primers used for amplification were a 5' forward primer (5'-GTGCCCTATGACACGGATCTA-3') and a 3' reverse primer (5'-AGTCCCAGTAATGGTCGCTAT-3'). The *FLT3* primers were a 5' forward primer (5'-AGGGACAGTGTAC GAAGCTG-3') and a 3' reverse primer (5'-GCTGTGCTTAA AGACCCAGAG-3'). The *CSF3R* primers were a 5' forward primer (5'-TCAAGTTGGTGCTATGGCAAGGCT-3') and a 3' reverse primer (5'-TTCTGCTTGATGATGCAGGAGGCT-3'). The *CSF2RA* primers were a 5' forward primer (5'-ATGT CACCGTACGTTGCAACACGA-3') and a 3' reverse primer (5-TGGGCTCAGAGCTTGGAAAGTTGT-3'). The *GAPDH* primers were 5'-ACAACTTTGGTATCGTGGAAGG-3' (5' forward primer) and 5'-GCCATCACGCCACAGTTTC-3' (3' reverse primer). The PCR conditions were 98˚C for 10 sec, followed by 55˚C for 30 sec and 72˚C for 1 min, for 35 cycles. After amplification, the PCR products were separated on a 2% agarose gel to confirm their abundance and size.

Quantitative PCR (qPCR) was performed using a SYBR Premix Ex Taq™ II (Takara) kit and a LightCycler 480 II amplifier (Roche, Basel, Switzerland), using the instructions of the manufacturer. The CEBPA primers were a 5' forward primer (5'-TGTATACCCCTGGTGGGAGA-3') and a 3' reverse primer (5'- TCATAACTCCGGTCCCTCTG-3'). The GAPDH primers were 5'- ACAACTTTGGTATCGTGGAAGG-3' (5' forward primer) and 5'-GCCATCACGCCACAGTTTC-3' (3' reverse primer). The PCR conditions were as follows: preincubation at 95˚C for 30 sec, 1 cycle; amplification at 95˚C for 5 sec, 60˚C for 30 sec, 40 cycles; melting at 95˚C for 10 sec, 65˚C for 60 sec, 1 cycle; cooling at 40˚C for 30 sec, 1 cycle. Relative expression level = $2^{-\Delta\Delta Ct}$, where ΔCt = Ct(gene of interest) - Ct(housekeeping gene), $\Delta\Delta Ct$ = ΔCt(test group) - ΔCt(control group).

Western blot analysis. Equal amount of cells was lysed using a radio-immunoprecipitation assay (RIPA buffer; Beyotime Institute of Biotechnology, Haimen, China) with phenylmethane sulfonyl fluoride (PMSF). Cell lyses were boiled for 10 min at 100˚C after adding sample buffer and separated using SDS-polyacrylamide gel electrophoresis. The target proteins were transferred onto nitrocellulose (NC) membranes and detected with specific primary antibody, followed by a IRDye800/700-conjugated secondary antibody against rabbit/mouse antibodies (LI-COR Biosciences, Lincoln, NE, USA). Primary antibodies included rabbit monoclonal antibody against mouse monoclonal antibody against Akt (1:1,000; Cell Signaling Technology, Danvers, MA, USA), eIF2α (1:1,000; Cell Signaling Technology), GAPDH (1:1,500; Cell Signaling Technology) and β-actin (1:1,500; Cell Signaling Technology), rabbit monoclonal antibody against C/EBP-α (1:1,000; Cell Signaling Technology), p-Akt (1:1000; Cell Signaling Technology), p-eIF2α (1:1,000; Cell Signaling Technology) and 4E-BP (1:1,000; Cell Signaling Technology).

Statistical analysis. Data were expressed as the mean ± SD of at least three independent experiments, and each group had three repetitions. Statistical significance was analyzed using the t-test. $P<0.05$ was considered significant. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses of the data.

Results

NB4-R1 cells are stably resistant to ATRA-induced myeloid differentiation and proliferation inhibition. To confirm the resistance of NB4-R1 cells to ATRA, we treated NB4-R1 and NB4 cells with ATRA or the same volume of the solvent DMSO as a control, respectively. CD11b expression of each group of cells was detected using FCM at the 72 h after treatment. NB4 cells showed high sensitivity to ATRA treatment. The percentage of CD11b positive cells among the NB4 cells which were treated with 1 μM ATRA and the DMSO control were 83.9±4.1 and 0.5±0.3%, respectively ($P<0.001$). ATRA almost achieved maximum of its efficiency on this concentration. Increasing the ATRA concentration to 10 μM did not further increase the percentage of CD11b positive cells (84.9±3.9%) of NB4 cells (Fig. 1A and C). NB4-R1 cells did not respond well to ATRA treatment. The percentage of CD11b positive cells among the NB4-R1 cells did not change when cells were treated with 1 μM ATRA (1 μM ATRA compared with DMSO control, 0.5±0.3 vs. 0.7±0.3%, $P>0.05$) and was only 2.0±0.6% after treatment with 10 μM ATRA ($P>0.05$) (Fig. 1B and C).

We then used the MTT assay to assess the proliferative ability of NB4 and NB4-R1 cells treated with ATRA. Proliferation curves of each group of cells were plotted (Fig. 1D and E). The concentrations at 50% inhibition (IC_{50}) of ATRA were calculated based on the logit regression line. The estimated IC_{50} value of ATRA for NB4 cells was 5.028 μM (95% confidence limits, 3.624-7.189) and for NB4-R1 cells was 42.030 μM (95% confidence limits, 28.373-105.403). The calculated resistance index of NB4-R1 cells to ATRA was 8.36. These results indicated that NB4-R1 cells were stably resistant to ATRA on differentiation inducing and cell growth arrest.

Expression of C/EBPα is strongly suppressed in ATRA-resistant NB4-R1 cells. NB4 and NB4-R1 cells in log growth phase were collected and the C/EBPα expression of each group of cells was detected using RT-qPCR and western blots. RT-qPCR showed that the C/EBPα mRNA levels

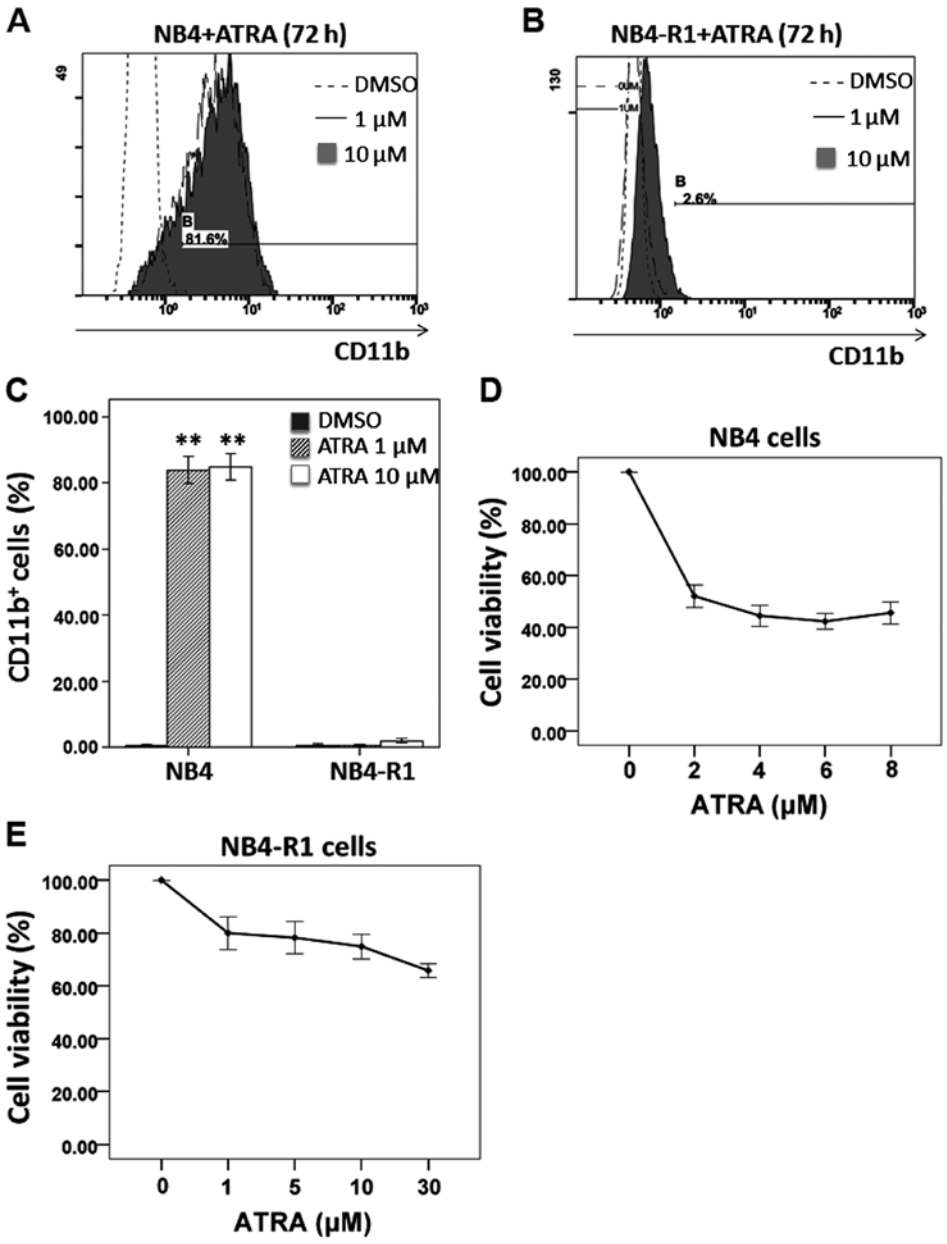

Figure 1. NB4-R1 cells were stably resistant to ATRA-induced myeloid differentiation and proliferation inhibition. CD11b expression of (A) NB4 and (B) NB4-R1 cells treated with 1 μM (solid line) or 10 μM ATRA (filled) or DMSO as a control (dotted line), for 72 h, were detected with flow cytometry (FCM). One representative FCM experiment is shown. Mean percentage of $CD11b^+$ cells was calculated (C). Each value represents the mean ± SD of three independent experiments. NS, $P>0.05$; $^{**}P<0.01$. Available cells of (D) NB4 and (E) NB4-R1 treated with 1 μM ATRA or DMSO for 72 h were evaluated by MTT assay. Each value represents the mean ± SD of three independent experiments.

were attenuated, both for NB4 and NB4-R1 cells, compared with PBMC from healthy donor subjects. However, there was no difference in mRNA levels between these two cell lines (Fig. 2A). When we performed western blots to detected protein level of C/EBPα, we found that NB4-R1 cells exhibited a more pronounced decrease in C/EBPα protein level than NB4 cells. Moreover, the inhibition in C/EBPα P42 was more significant than the P30 form, and expression of P42 C/EBPα was almost totally abolished in NB4-R1 cells, resulting in a significantly decreased P42/P30 ratio (Fig. 2B).

PI3K/Akt/mTOR signaling pathway inhibition and eIF2α kinase activation are responsible for the C/EBPα suppression in NB4-R1 cells. To identify the possible mechanisms of C/EBPα suppression in NB4-R1 cells, we characterized the activation of signaling pathways associated with C/EBPα modulation in NB4 and NB4-R1 cells. Inhibition of the PI3K/AKT/mTOR signalling pathway as evidenced by a reduction in Akt phosphorylation showed in NB4-R1 cells (Fig. 2C). To test whether PI3K/Akt/mTOR pathway inhibition was sufficient for C/EBPα suppression, we treated NB4 cells with the PI3K inhibitor LY294002 and the mTOR inhibitor RAD001 (everolimus) respectively. When NB4 cells were treated with 10 μM LY294002 for 48 h, C/EBPα expression was significantly decreased with deactivation of Akt, compared with the DMSO control. The expression of C/EBPα further decreased when the LY294002 concentration was increased to 20 μM (Fig. 2D). Similarly, suppression of C/EBPα was accompanied

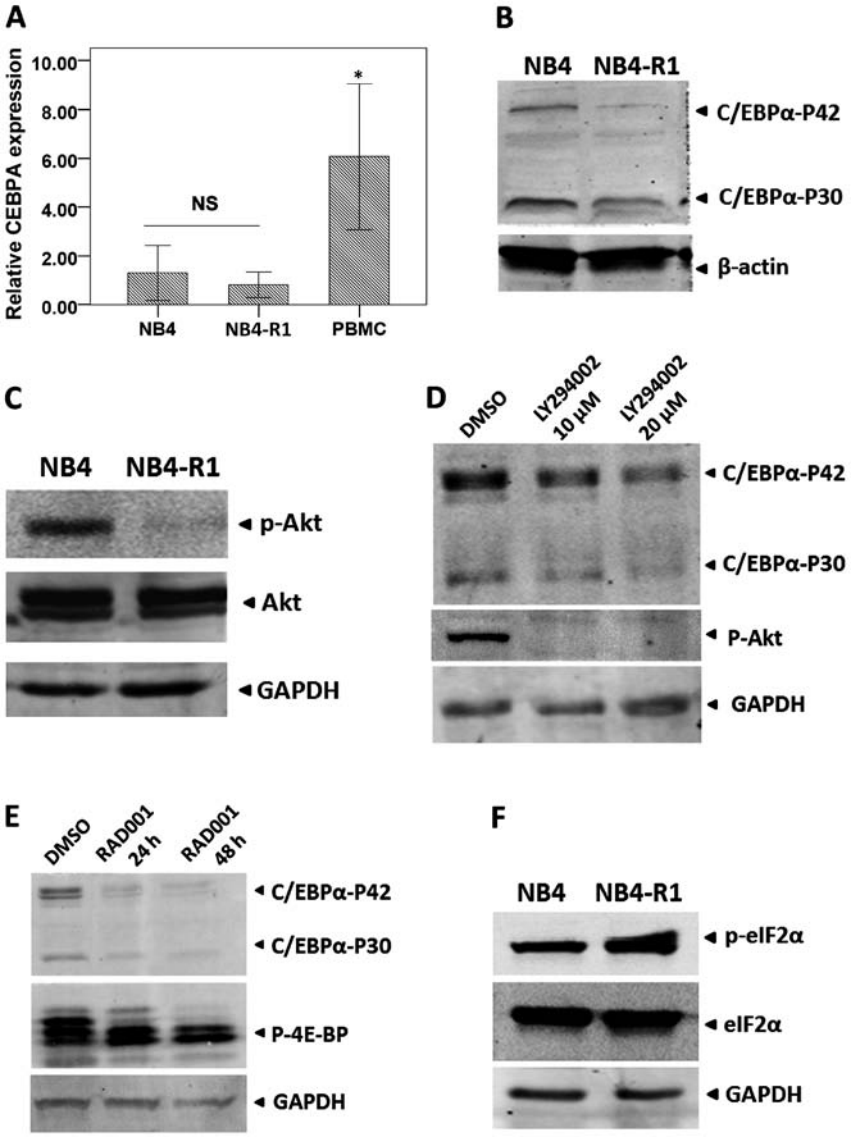

Figure 2. Inhibition of the PI3K/Akt/mTOR pathway and activation of eIF2α kinase were responsible for the C/EBPα suppression in NB4-R1 cells. (A) The mRNA level of the *CEBPA* gene in NB4 and NB4-R1 cells and of healthy volunteers was determined by RT-qPCR. Each value represents the mean ± SD of three independent experiments. (B) C/EBPα P42 and P30 protein levels in NB4 and NB4-R1 cells were detected by western blots. Left panel, NB4 cells; right panel, NB4-R1 cells. (C) Phosphorylation level of Akt in NB4-R1 cells was l detected by western blots. Left panel, NB4 cells; right panel, NB4-R1 cells. (D) C/EBPα expression and phosphorylation level of Akt were measured by western blot analysis afer NB4-R1 cells were treated with 10 μM or 20 μM LY294001. (E) C/EBPα expression and phosphorylation level of 4E-BP were measured by western blot analysis afer NB4-R1 cells were treated with 10 nM RAD001 for 24 and 48 h. (F) The phosphorylation level of eIF2α in NB4 and NB4-R1 cells. Left panel, NB4 cells; right panel, NB4-R1 cells. Similar results were obtained in three independent experiments.

by deactivation of eukaryotic translation initiation factor (eIF4E)-binding proteins (4E-BP), a substrate of mTOR, when NB4 cells were treated with 10 nM RAD001 for 24 h. More pronounced suppression of C/EBPα was induced by extending time of treatment to 48 h (Fig. 2E).

The eIF2α kinase is a translation regulator whose phosphorylation has been reported to cause a decreased C/EBPα P42/P30 ratio in HL60 cells (32). In the present study, we found that eIF2α phosphorylation was enhanced in NB4-R1 cells compared with NB4 cells (Fig. 2F). These results explained, at least partially, why a decrease of full-length C/EBPα was more pronounced than that of P30.

Restoration of C/EBPα P42, but not P30, induces differentiation of NB4-R1 cells. To study the possible involvement of C/EBPα suppression in the differentiation block of NB4-R1 cells, we restored C/EBPα P42 and P30 using lentivirus vectors. Western blots verified the overexpression of C/EBPα P42 or P30 NB4-R1 cells after infection and consequent positive selection (Fig. 3A). The basic lentivirus vector was used as a control. CD11b expression was increased by 20.1% in NB4-R1 cells with restored P42, compared with the vector control (P42 compared with vector control, 24.2±3.7 vs. 4.1±0.4%, respectively, P<0.01). However, restoring P30 did not increase CD11b expression in NB4-R1 cells (P30 compared with the vector control, 7.7±1.1 vs. 4.1±0.4%, P>0.05). Similarly, CD14 expression increased by 25.4% in NB4-R1 cells with restored P42, but not with restoration of P30, compared with the vector control (P42 compared with the vector control, 34.7±1.3 vs. 9.3±0.2%, P<0.01). Restoration of P30 even slightly decreased

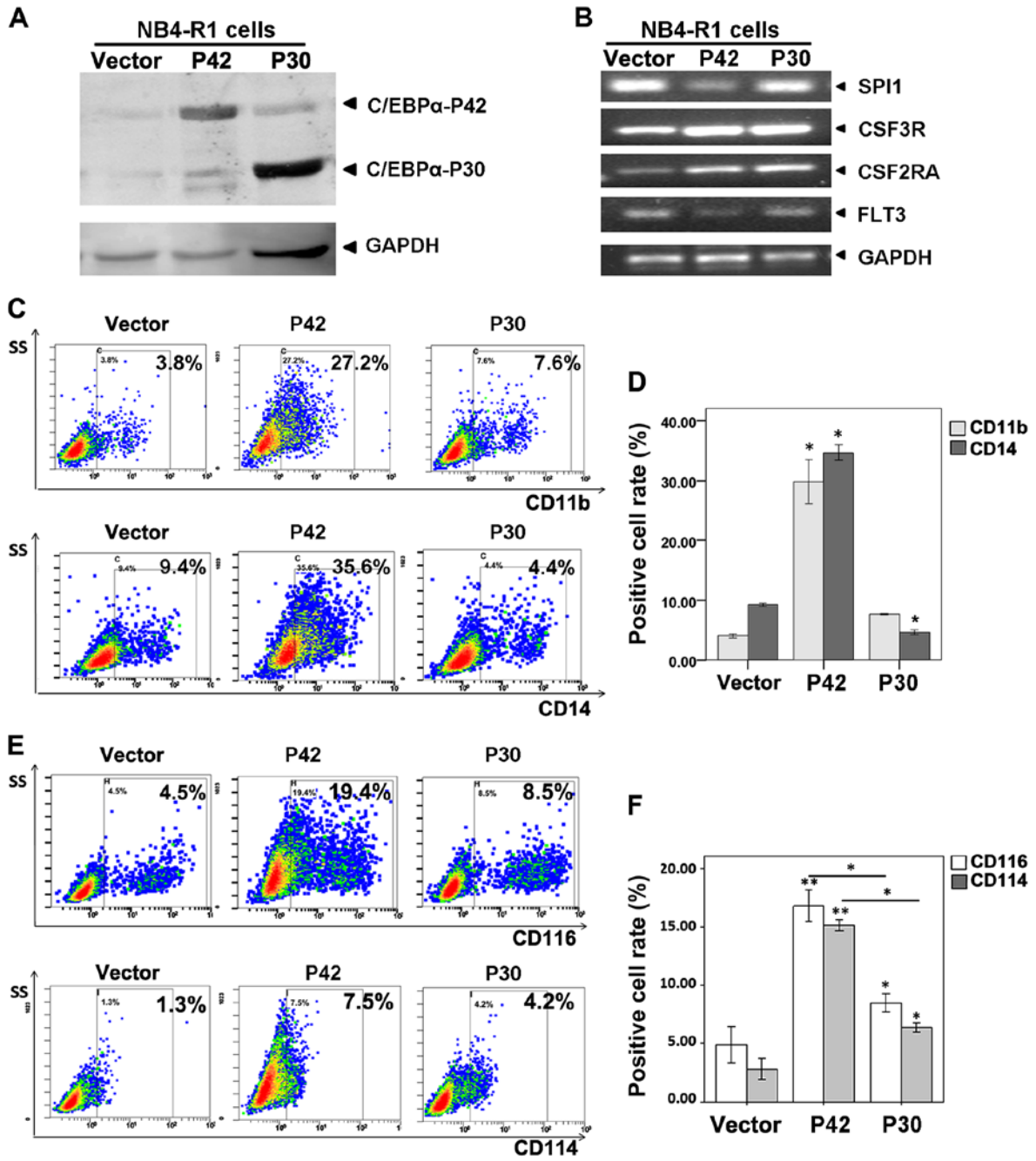

Figure 3. C/EBPα P42, but not P30 restoration, induces the differentiation of NB4-R1 cells. (A) C/EBPα protein levels in NB4-R1 cells following transfection with recombinant C/EBPα P42 or P30 expressing lentivirus vectors. Lane 1, lentivirus vector control group; lane 2, C/EBPα P42 overexpressing group; lane 3, C/EBPα P30 overexpressing group. (B) The mRNA levels of the CSF3R, CSF2RA, SPI1 and FLT3 genes in NB4-R1 cells infected with control (panel 1) or C/EBPα P42 expressing (panel 2) or P30 expressing (panel 3) lentivirus vectors. (C) CD11b and CD14 (E) CD116 and CD114 expression of NB4-R1 cells infected with vector control or C/EBPα P42 expressing or P30 expressing lentivirus vectors. The result of a representative FCM experiment is shown. (D) Mean percentage of CD11b, CD14 and (F) CD116 and CD114 positive cells were calculated. Each value represents the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. *P<0.05, **P<0.01.

CD14 expression in NB4-R1 cells (P30 compared with the vector control, 4.7±0.4 vs. 9.3±0.2%, P<0.05) (Fig. 3C and D).

We then detected G-CSFR (CD114) and GM-CSFR (CD116) expression on the surface of NB4-R1 cells with restored C/EBPα P42 or P30. NB4-R1 cells with restored P42 had a 12.3% increase of CD114 expression compared with the vector control (P42 compared with the vector control, 15.1±0.5 vs. 2.8±0.9%, P<0.01), while cells with restored P30 only had a 3.8% increase (P30 compared with the vector control, 6.4±0.4 vs. 2.8±0.9%, P<0.05). NB4-R1 cells with restored P42 had a 12.3% increase of CD116 expression compared with the vector control (P42 compared with the vector control, 26.1±2.0 vs. 5.6±1.6%, respectively, P<0.01), while cells with restored P30 only had a 3.6% increase (P30 compared with the vector control, 9.4±0.6 vs. 5.6±1.6%, respectively, P<0.05). Although the expression of CD114 and CD116, respectively, in cells with restored P30 was higher than in cells infected with the vector control, both were still

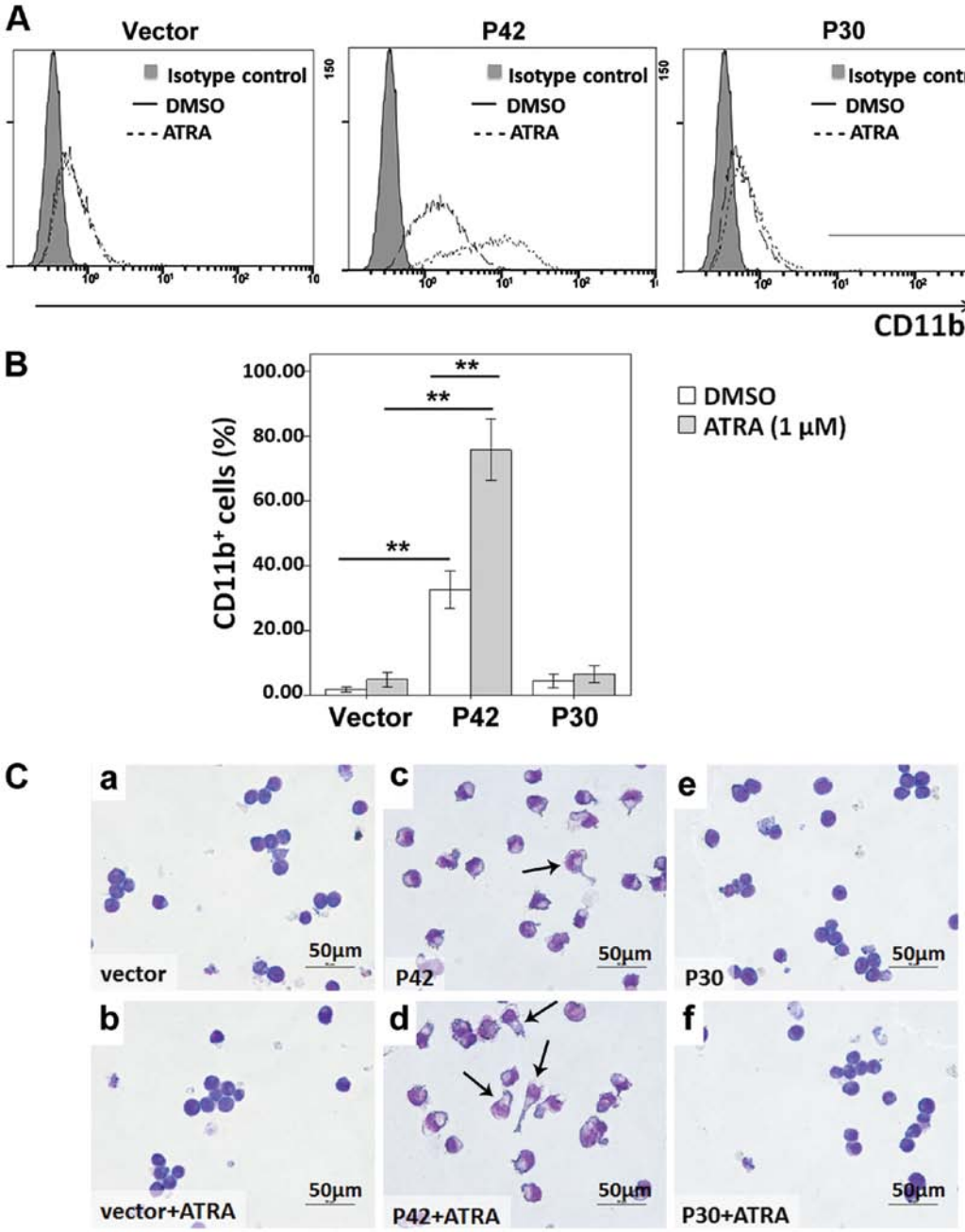

Figure 4. Restoration of C/EBPα P42, but not P30, overcome ATRA resistance in NB4-R1 cells. (A) CD11b expression before or after 1 μM ATRA treatment in NB4-R1 cells infected with control (left) or C/EBPα P42 expressing (middle) or P30 expressing (right) lentivirus vectors. A representative flow cytometry experiment is shown. (B) Mean percentage of CD11b positive cells was calculated **P<0.01. Each value represents the mean ± SD of three independent experiments. (C) Morphologic changes with DMSO (upper rank) or 1 μM ATRA (below rank) treatment for 72 h in NB4-R1 cells transfected with C/EBPα P42 or P30 or control vector were observed by Giemsa staining. (a) Control vector infection followed by DMSO treatment. (b) Control vector infection followed by 1 μM ATRA treatment. (c) Restoration of C/EBPα P42 followed by DMSO treatment. (d) Restoration of C/EBPα P42 followed by 1 μM ATRA treatment. (e) Restoration of C/EBPα P30 followed by DMSO treatment. (f) Restoration of C/EBPα P42 followed by 1 μM ATRA treatment. Similar results were obtained in three independent experiments.

significantly lower than in cells with restored P42 (P<0.01) (Fig. 3E and F).

C/EBPα P42 and P30 exert different regulatory effects on differentiation- and proliferation-associated genes in NB4-R1 cells. The mRNA levels of myeloid differentiation-related genes were detected by PCR after C/EBPα P42 or P30 were restored in NB4-R1 cells. Restoration of C/EBPα P42 and P30 equally upregulated transcription of *CSF3R* gene and *CSF2RA* gene (G-CSFR and GM-CSFR encoding genes, respectively) in NB4-R1 cells. However, restoration of P42, but not P30, decreased the mRNA level of *SPI1* (PU.1-encoding gene) and *FLT3* gene (Fig. 3B). These results indicated that C/EBPα P42 induced myeloid differentiation by upregulating the *CSF3R* and *CSF2RA* genes and by downregulating the *SPI1* and *FLT3* genes. P30 only retained the ability to regulate the *CSF3R* and *CSF2RA* genes, which was not sufficient to induce myeloid differentiation of NB4-R1 cells.

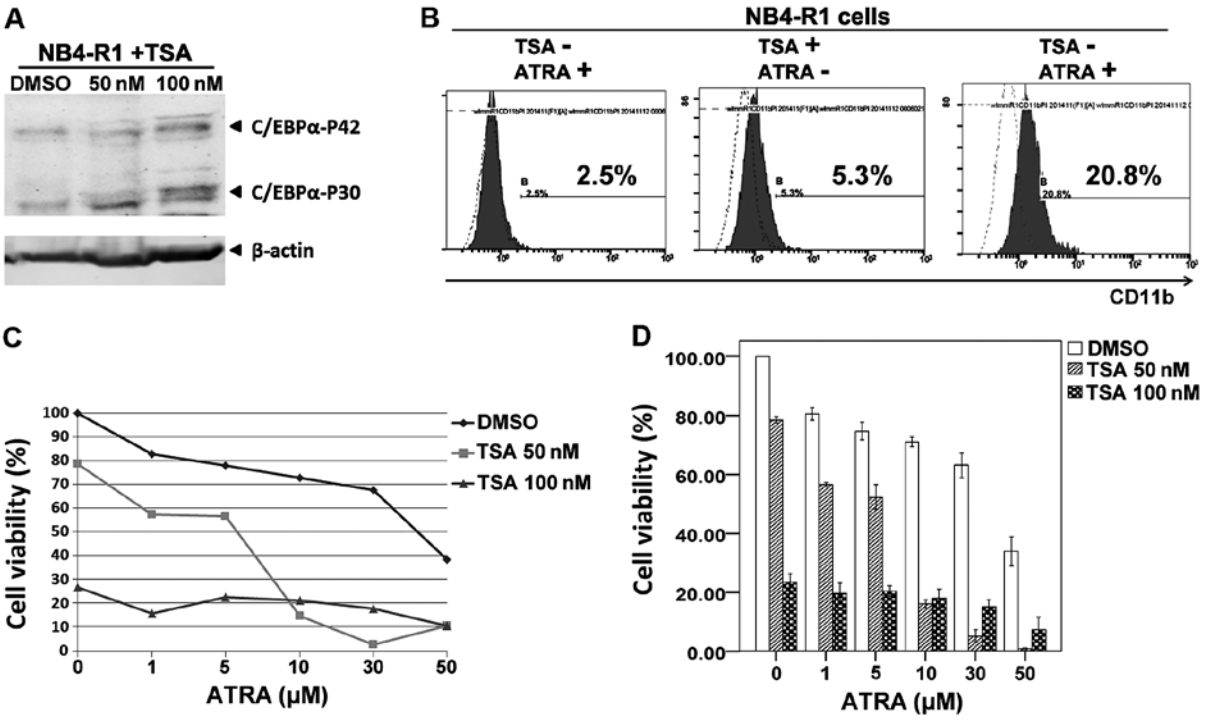

Figure 5. TSA upregulates C/EBPα expression and thereby exhibits a synergistic effect with ATRA in inducing differentiation and cell growth arrest. The expression level of C/EBPα was determined by western blot analysis after NB4-R1 cells were treated with dimethylsulphoxide (DMSO) control (panel 1) or 50 nM (panel 1) or 100 nM (panel 1) trichostatin (TSA) for 24 h. (B) CD11b expression was determined by flow cytometric analysis after NB4-R1 cells were treated with 50 nM TSA or 1 μM all-*trans* retinoic acid (ATRA) alone or combined for 48 h. Dotted line represents CD11b expression of cells treated with DMSO control. Viable cells were detected by the MTT assay. (C) Proliferation curves and (D) histograms are shown. Data are representative of the mean ± SD of three independent repeated experiments.

Restoration of C/EBPα P42, but not P30, overcome ATRA resistance in NB4-R1 cells. In order to confirm the association of C/EBPα suppression and ATRA resistance of NB4-R1 cells, we treated NB4-R1 cells with 1 μM ATRA or DMSO for 72 h after C/EBPα P42 or P30 expressing vector or control vector were stably transduced. Due to the differentiation inducing effect of C/EBPα P42 itself, CD11b expression increased by 30.6% in NB4-R1 cells with restored C/EBPα P42 compared with the vector control in ATRA-free group (P42 compared with the vector control, 32.6±5.9 vs 1.8±0.8%, P<0.01). More evident increase in CD11b expression was shown in NB4-R1 cells with combined C/EBPα P42 restoration and ATRA treatment (P42 compared with the vector control, 75.7±9.4 vs. 4.9±2.3%, P<0.01). However, restoration of P30 did not increase CD11b expression with or without ATRA treatment (DMSO and 1 μM ATRA treatment, 4.5±2.2 vs. 6.6±2.6%) (Fig. 4A and B).

Morphological changes of NB-R1 cells were observed using light microscopy after Giemsa staining. We found that cells infected with the control vector showed classic features of APL cells, including deeply stained nuclei, a large nucleus/cytoplasm ratio, and colony formation. ATRA treatment did not alter morphology of these cells. Morphological alterations were observed in NB4-R1 cells after restoring C/EBPα P42, such as faded nuclear staining and a decreased nucleus/cytoplasm ratio. These alterations were more obvious in cells with combined restoration of P42 and ATRA treatment. A part of NB4-R1 cells differentiated into macrophage-like cells, which grew with adherence and pseudopodia. However, C/EBPα P30 restoration and subsequent ATRA treatment did not induce any morphological alteration in NB4-R1 cells (Fig. 4C). These results indicated that suppression of C/EBPα P42, but not P30, was associated with ATRA resistance in NB4-R1 cells. Restoring C/EBPα P42 expression could enhance the differentiation induced by ATRA in NB4-R1 cells.

TSA upregulates C/EBPα expression and thereby exhibits a synergistic effect with ATRA in inducing differentiation and inhibiting proliferation. C/EBPα expression in NB4-R1 cells was detected after treatment with different concentrations of the histone deacetylase inhibitor (HDACi) trichostatin (TSA). Expression of C/EBPα was upregulated after treatment with 50 nM TSA for 24 h, and further increased when the TSA concentration was increased to 100 nM (Fig. 5A). To confirm the effect of C/EBPα upregulation induced by TSA on differentiation and ATRA sensitivity of NB4-R1 cells, we treated NB4-R1 cells with 50 nM TSA alone or combined with 1 μM ATRA. CD11b expression was detected by FCM at 48 h. For the same reasons that C/EBPα induced differentiation, CD11b expression was slightly increased after treatment with 50 nM TSA alone. When NB4-R1 cells were treated with 50 nM TSA combined with 1 μM ATRA, CD11b expression was significantly upregulated (Fig. 5B).

The MTT assay was then used to determine the effect of TSA combined with ATRA in growth arrest. Viable cells decreased by 25.6% when cells were treated with 1 μM ATRA combined with 50 nM TSA, compared with cells treated with 1 μM ATRA alone, and by 58.4% when cells were treated with 10 μM ATRA combined with 50 nM TSA. However, 100 nM

TSA caused a severe toxic reaction in NB4-R1 cells, which resulted in low cell viability (Fig. 5C and D). These results indicated that ATRA combined with 50 nM TSA significantly enhanced the proliferation inhibition in NB4-R1 cells.

Discussion

C/EBPα is one of the most important transcription factors for myeloid development. Suppression of C/EBPα has been detected in many subtypes of AML with chromosome abnormalities, including M2 with AML1-ETO, M3 with PML-RARα and M4 with CBFB-MYH11. Corepressor complexes recruited by fusion proteins were the main reported reasons for suppression of C/EBPα in these subtypes of AML (33-37). In the present study, C/EBPα suppression was more pronounced in the ATRA resistant APL NB4-R1 cells than in ATRA sensitive NB4 cells. Furthermore, suppression of the C/EBPα P42 isoform was greater than the P30 isoform. The P42 isoform of C/EBPα was almost absent in NB4-R1 cells.

Because C/EBPα plays an essential role in myeloid differentiation, we postulated that C/EBPα P42 suppression is associated with ATRA resistance in NB4-R1 cells. To confirm this possibility, we restored C/EBPα P42 and P30 levels in NB4-R1 cells using lentivirus vectors. As expected, restoration of C/EBPα P42 resulted in differentiation of NB4-R1 cells. Furthermore, restoration of C/EBPα P42 enhanced ATRA sensitivity of NB4-R1 cells. This result suggested that C/EBPα P42 was a key molecule in the differentiation inducing effect of ATRA as a vital transcription factor controlling expression of a series of myeloid differentiation associated genes. Suppression of C/EBPα P42 interrupted the differentiation process initiated by ATRA in APL cells. The recovery of ATRA sensitivity was dependent on the first transcriptional activation domain (TAD1) of C/EBPα at N-terminus. C/EBPα P30, which lacked TAD1, did not increase CD11b expression or induce morphological changes after treatment with ATRA. P30 reserved DNA binding domain of C/EBPα at C-terminus. It committed a competitive inhibition to P42 in a dominant-negative manner (27). In the present study we found that overexpression of P30 slightly decreased CD14 expression instead of increasing it.

Restoration of C/EBPα P42 overcame the myeloid differentiation block, by upregulating the *CSF3R* and *CSF2RA* genes and downregulating the *SPI1* gene. The *SPI1* gene encodes transcription factor PU.1, which is essential in monocyte-macrophage differentiation (38-40). Friedman *et al* (41) demonstrated that C/EBPα activates the murine PU.1 promoter in myeloid progenitor 32Dcl3 cells. Our results suggested that in later stage of myeloid differentiation, C/EBPα favored granulocytes over monocytes by suppressing PU.1 transcription. *FLT3* gene, whose production of FMS-like tyrosine kinase 3 (FLT3) was associated with proliferation of hematopoietic cells, was also suppressed by P42 restoration. Constitutive activation of FLT3 was reported to be involved in the pathogenesis of AML and acute lymphoblastic leukemia (ALL) (42,43). Its suppression may be associated with growth arrest of APL cells after P42 restoration. However, P30 only retains the ability to regulate the *CSF2RA* and *CSF3R* genes, which was not sufficient to induce myeloid differentiation of NB4-R1 cells.

Both NB4 and NB4-R1 cells showed significantly lower C/EBPα mRNA levels compared with healthy controls, but C/EBPα transcription levels between these two cell lines were equal. This indicated that the severe suppression of C/EBPα in NB4-R1 cells was not at the level of transcription. Activation of translational and post-translational modification pathways in NB4 and NB4-R1 cells was then determined. Decreased phosphorylation of PI3K/Akt/mTOR signaling pathway, as evidenced by a reduction in Akt phosphorylation, was found in NB4-R1 cells. when we treated NB4 cells with PI3K/Akt/mTOR inhibitors LY294002 and RAD001, respectively, expression of C/EBPα decreased as expected. Our results confirmed the relationship of the suppressed PI3K/Akt/mTOR pathway with decreased C/EBPα expression in NB4-R1 cells. The activation level of eIF2α, which was reported to be associated with upregulation of C/EBPα p30/P42 ratios (32), was increased in NB4-R1 cells. This result at least partly explained the different suppression levels of C/EBPα P42 and P30 in NB4-R1 cells.

Histone deacetylase recruits target genes using PML-RARα of APL cells, and exerts a negative regulatory effect on these types of genes. HDACi valproic acid (VPA) was reported to relieve downstream gene expression and have a synergistic effect with ATRA in inducing differentiation in NB4 cells (40). However, the effects of HDACi on ATRA-resistant NB4-R1 cells remain unknown. We found that HDACi TSA was capable of increasing C/EBPα expression in NB4-R1 cells. Differentiation was successfully induced by ATRA when the C/EBPα expression was restored by TSA. TSA showed a synergistic effect with ATRA in inducing myeloid differentiation and arresting cell growth. This synergistic effect demonstrated the important role of C/EBPα suppression in ATRA resistance as well. However, TSA equally upregulated expression levels of C/EBPα P42 and P30. Increases in the dominant negative form P30 limited the differentiation induction effects of TSA and ATRA. Thus, agents that specifically upregulate P42 and subsequently restore the ATRA sensitivity of APL cells still need to be identified. Also, more detailed studies on the original cause of PI3K/Akt/mTOR inhibition and associated C/EBPα suppression are required.

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