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Combined staurosporine and retinoic acid induces differentiation in retinoic acid resistant acute promyelocytic leukemia cell lines

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All-trans retinoic acid (ATRA) resistance has been a critical problem in acute promyelocytic leukemia (APL) relapsed patients. In ATRA resistant APL cell lines NB4-R1 and NB4-R2, the combination of staurosporine and ATRA synergized to trigger differentiation accompanied by significantly enhanced protein level of CCAAT/enhancer binding protein ϵ (C/EBP ϵ) and C/EBP β as well as the phosphorylation of mitogen-activated protein (MEK) and extracellular signal-regulated kinase (ERK). Furthermore, attenuation of the MEK activation blocked not only the differentiation but also the increased protein level of C/EBP ϵ and C/EBP β . Taken together, we concluded that the combination of ATRA and staurosporine could overcome differentiation block via MEK/ERK signaling pathway in ATRA-resistant APL cell lines.

Since the introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) in the conventional chemotherapy of acute promyelocytic leukemia (APL), the prognosis of APL has significantly improved, approximately 90% of patients achieved 5-year disease free remission¹. However, ATRA resistance has still been a critical problem in relapsed patients, especially the patients consolidated with ATRA-containing treatment. Several mechanisms of ATRA resistance have been speculated by early studies including an increased oxidative catabolism of ATRA by cytochrome P450 enzymes² or P-glycoprotein³, reduced cellular ATRA concentration by an enhanced level of cellular RA-binding protein (CRABP)^{4,5}. However, more and more clinical observations and *in vitro* studies confirmed that the defects of the pathogenic fusion gene of APL, *promyelocytic leukemia-retinoid acid receptor α* (*PML-RAR α*), particularly genetic mutations in the ligand binding domain (LBD) of the *RAR α* region resulting in deficient ATRA binding was the major cause of ATRA resistance^{6–15}. Furthermore, altered ligand-induced co-repressor release, co-activator recruitment and impaired transcriptional activation of genes with the retinoic acid response elements (RAREs) sites were associated with ATRA resistance^{16,17}. Besides *PML-RAR α* , mutations in other genes, such as *FLT3-ITD* or *TP53* might also contribute to ATRA resistance^{18,19}.

As_2O_3 was demonstrated to be effective in the treatment of relapsed APL patients, achieving complete remission (CR) rate of more than 80%²⁰. Although adverse effects of As_2O_3 were relatively rare, it still had severe side effects with long-term use. Moreover, clinical resistance to As_2O_3 was also observed²¹. Histone deacetylase (HDAC) enzymes are the essential components of the co-repressor complex that promoted chromatin condensation and reduced gene transcription. HDAC inhibitor was confirmed to restore sensitivity to ATRA by *in vitro* study²². However, clinical trial showed that the addition of HDAC inhibitor to ATRA was only effective in very limited number of ATRA-resistant APL patients^{12,23}. Since HDAC has a dual role in tumorigenesis, behaving as oncosuppressor during tumor initiation while performing as oncogene in tumor maintenance, clinical use of HDAC inhibitor should take in caution²⁴. Allogeneic bone marrow transplantation was another therapy choice, but only applicable to small amount of patients. Therefore, new therapy approaches to ATRA resistant APL patients were required to be developed. Indeed, other agents such as cAMP, granulocyte colony-stimulating factor, tumor necrosis factor, oridonin, tyrosine kinase inhibitor STI571 and interferon- γ have been shown to cooperate with ATRA to induce differentiation in ATRA resistant APL cells^{25–30}.

Protein kinase C (PKC) is a family of serine/threonine kinases, consisting of 13 isozymes which play a crucial role in signaling transduction of various cell functions including proliferation, differentiation, apoptosis, cell



migration and gene expression. Intensive studies has explored its contribution to carcinogenesis and rendered it an attractive target for cancer therapy³¹. ATRA either activated or suppressed certain PKC isozyme activity in different cell lines^{32–35}. In APL cells, PKC δ was activated by ATRA. However, the role of activated PKC δ in ATRA-induced differentiation in APL cells was quite controversial^{36,37}. Activated PKC δ was confirmed to be required for ATRA-induced differentiation³⁶. On the contrary, McNamara S et al³⁷ demonstrated that activated PKC δ could increase protein stability and activity of topoisomerase II β , resulting in RAR α transcription repression and RA resistance. Another PKC isoform, PKC α was suggested to negatively modulate terminal neutrophil differentiation³⁸.

To investigate whether inhibition of PKC could reverse ATRA resistance, we explored the effect of the combination of staurosporine, one of the most powerful but less specific PKC inhibitors with ATRA in some ATRA-resistant APL cell lines, NB4-R1 and NB4-R2. Neither staurosporine nor ATRA could trigger differentiation in ATRA-resistant APL cell lines. Surprisingly, the combination of ATRA and staurosporine could overcome retinoid resistance in these cell lines. The protein level of CCAAT/enhancer binding protein ϵ (C/EBP ϵ) and C/EBP β as well as the phosphorylation of mitogen-activated protein (MEK) and extracellular signal-regulated kinase (ERK) was enhanced by the combined treatment. Moreover, MEK/ERK signaling pathway was demonstrated to be required for the combination-induced differentiation.

Results

Combination of staurosporine and ATRA induced granulocytic differentiation accompanied by proliferation inhibition in NB4-R1 and NB4-R2 cells. To investigate the effect of combined treatment of staurosporine and ATRA on NB4-R1 and NB4-R2 cells, we first tested the concentration of staurosporine studied in both cell lines. DMSO treatment was regarded as control since both ATRA and staurosporine were dissolved in it. 2 nM staurosporine was determined to be used since there were no obvious effects on cell proliferation (Figure 1a and 1b) and survival at such concentration (Figure 1c and 1d). The combined treatment suppressed cell growth at the third day (Figure 1a and 1b). The proliferation inhibition rate calculated as Methods mentioned was $36.1 \pm 3.1\%$ for NB4-R1 cells and $34.5 \pm 2.7\%$ for NB4-R2 cells. However, cell viability maintained above 90% in these two cell lines with any treatment for 72 hours. Annexin-V assay also showed that more than 90% cells were PI negative and Annexin-V negative even with combined treatment (Figure 1c and 1d). Thus, the combined treatment had no effect on cell survival but only inhibited proliferation in these two cell lines.

Both cell lines had a characteristic morphology of APL blast such as irregular nucleus and large nuclear/cytoplasm ratio (Figure 1e and 1f). After incubated with 2 nM staurosporine or 1 μ M ATRA alone for 72 hours, no significantly morphological change was observed in NB4-R1 cells (Figure 1e). Meanwhile, most of the NB4-R2 cells retained the morphology of APL blast with any single treatment while some cells presented decreased nuclear/cytoplasm ratio with ATRA alone (Figure 1f). However, when simultaneously treated with staurosporine and ATRA for 72 hours, both cell lines displayed appearances of matured granulocytes, such as lobed nuclei accompanied by markedly decreased nuclear/cytoplasm ratio (Figure 1e and 1f). Nitroblue tetrazolium (NBT) reduction activity did not increase remarkably with any single treatment in both cell lines (Figure 1g). In addition, the expression of CD11b was somewhat enhanced with any single treatment in NB4-R1 and NB4-R2 cells (Figure 1h–1j). However, with the combination of staurosporine and ATRA, a more than additive effect was observed. The combined treatment significantly increased the percentage of CD11b positive cells in both cell lines (ST + RA compared with DMSO, $73.9 \pm 6.6\%$ vs $6.9 \pm 1.3\%$, $\chi^2 = 9321.63$, $P < 0.001$ in NB4-R1 cells and $75.1 \pm 2.3\%$ vs $9.0 \pm 1.6\%$, $\chi^2 = 8965.07$, $P < 0.001$ in NB4-R2 cells,

Figure 1h–1j). Accordingly, prominently enhanced NBT reduction activity was observed in both cell lines with combined treatment (ST + RA compared with DMSO, 0.129 ± 0.006 vs 0.014 ± 0.002 , $P = 0.0007$ in NB4-R1 cells and 0.161 ± 0.014 vs 0.018 ± 0.003 , $P = 0.0006$ in NB4-R2 cells, Figure 1g). Thus, these results demonstrated that the combined treatment induced granulocytic differentiation and indicated that the combination of staurosporine and ATRA could overcome maturation block in ATRA-resistant APL cells.

Modulation of C/EBPs and PML-RAR α as well as activation of MEK/ERK by the combined treatment. To explore the molecular mechanisms of combined treatment-induced differentiation, we first examined several proteins involved in granulocytic differentiation, especially which are critical for the promyelocyte to granulocyte transition. The induction of CCAAT/enhancer binding protein β (C/EBP β) and C/EBP ϵ expression is implicated in the later stage of granulocytic differentiation^{39,40}. Therefore, cells were treated with staurosporine, ATRA, or staurosporine plus ATRA for 24 hours and the protein levels of C/EBP β and C/EBP ϵ were detected by immunoblotting. As shown in Figure 2, in NB4-R1 cells, ATRA but not staurosporine elevated C/EBP β protein level while the cellular level of C/EBP ϵ was enhanced by both single treatment. In NB4-R2 cells, only ATRA treatment increased the protein level of C/EBP β and C/EBP ϵ . However, the up-regulation of C/EBP β and C/EBP ϵ was much more remarkable with the combined treatment in both cell lines (Figure 2). These results suggested that C/EBP β and C/EBP ϵ may be responsible for the differentiation effect of combined treatment in ATRA-resistant APL cells.

The degradation of PML-RAR α has been the crucial mechanism of ATRA-induced granulocytic differentiation of APL cells⁴¹. Treated with ATRA for 72 hours, the band corresponding to PML-RAR α was almost disappeared in NB4-R1 cells or decreased in NB4-R2 cells (Figure 2). Meanwhile, the protein level of RAR α was reduced in both cell lines with ATRA treatment (see Supplemental Fig. S1 online). Unexpectedly, the addition of staurosporine totally restored ATRA-mediated PML-RAR α alteration in both cell lines (Figure 2). Interestingly, in NB4-R2 cells, staurosporine alone could increase the protein level of RAR α and inhibit ATRA-induced reduction of RAR α (see Supplemental Fig. S1 online). These data indicated that the cooperation differentiation effect of ATRA and staurosporine was independent of the regulation of PML-RAR α .

Our previous study demonstrated that the non-genomic effect, MEK/ERK signaling pathway could positively regulate ATRA-induced differentiation in APL cells (unpublished data). To elucidate whether MEK/ERK signaling pathway was activated, the phosphorylated MEK and ERK1/2 were assessed by Western blot analysis in cells treated with ATRA or/and staurosporine for 24 hours. As illustrated in Figure 2, the combined treatment increased the amount of phosphorylation of MEK and ERK1/2 more significantly than that with any single treatment in both cell lines. The total amount of MEK and ERK1/2 in both cell lines remained almost unaltered after any treatment (Figure 2).

MEK/ERK activation was required for the combination-induced differentiation and the up-regulation of C/EBPs. Having shown that the combination phosphorylated MEK and ERK, we tested whether MEK/ERK activation was necessary for ATRA and staurosporine-mediated differentiation. Cells were incubated with 1 μ M U0126, a specific inhibitor of MEK for 1 hour prior to other treatments. The effectiveness of U0126 was evaluated by ERK1/2 phosphorylation. U0126 did attenuate ERK1/2 activation in these 2 cell lines (Figure 3a). Moreover, it abrogated cell differentiation triggered by the combined treatment in both cell lines as assessed by morphology (Figure 3b and 3c), NBT reduction assay (U + ST + RA compared with ST + RA, 0.026 ± 0.003 vs 0.129 ± 0.006 , $P = 0.0076$ in NB4-R1 cells, 0.045 ± 0.008 vs 0.161 ± 0.014 , $P = 0.0199$ in NB4-R2 cells Figure 3d) and CD11b expression (U + ST + RA

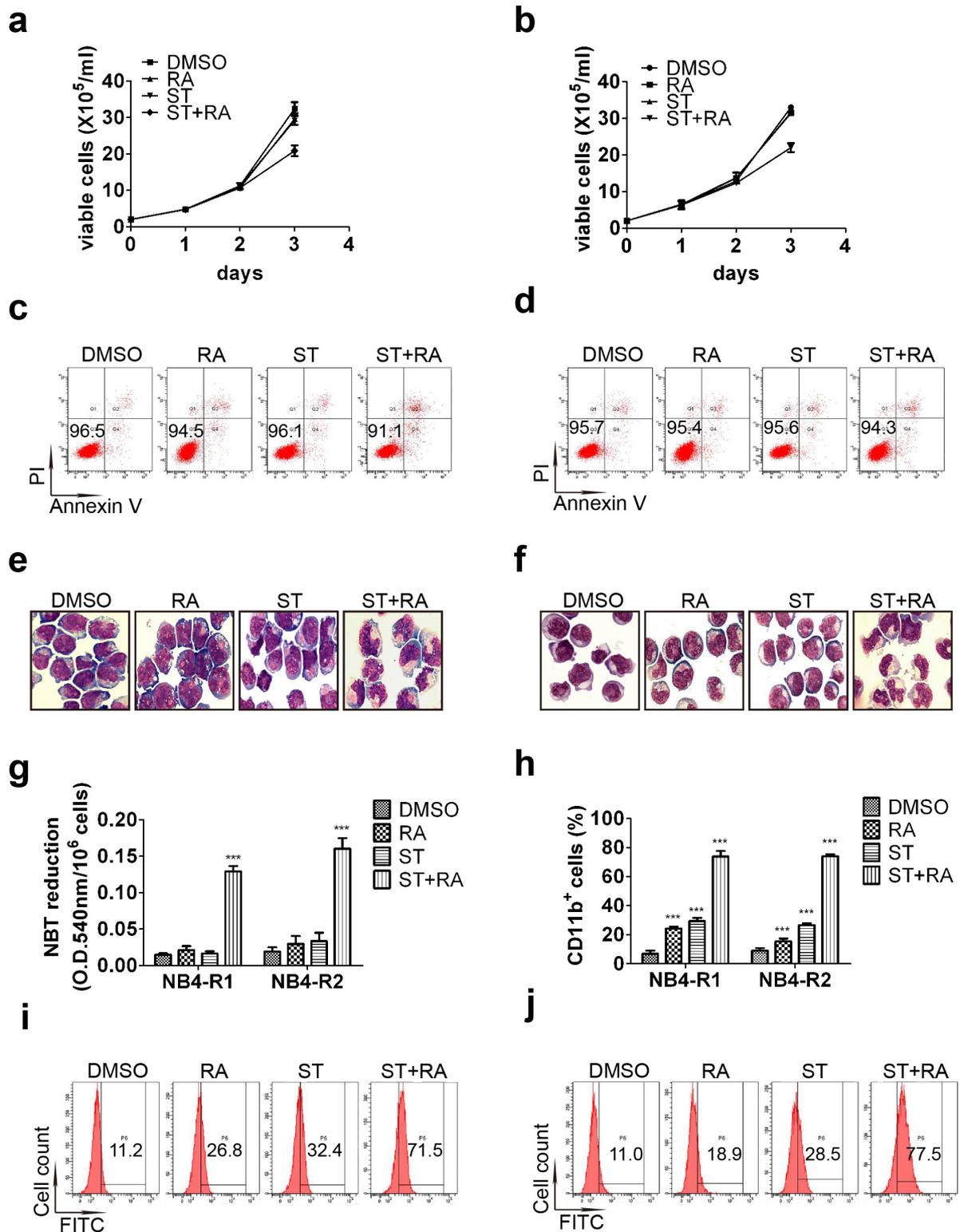


Figure 1 | Effects of combined treatment on the growth, survival and differentiation in NB4-R1 and NB4-R2 cells. Cell growth of NB4-R1 (a) and NB4-R2 (b) treated with 2 nM staurosporine(ST) or/and 1 μ M ATRA(RA) was calculated as mentioned in Methods. One representative experiment was shown. Each value represented the mean \pm SD of triplicate samples. Similar results were obtained in three independent experiments. Annexin-V assay of NB4-R1 (c) and NB4-R2 cells (d) treated with ST or/and RA for 72 hours. The percentages of viable (PI and Annexin V both negative) cells were shown in the corresponding panels. Results were representative among three independent experiments. Representative morphologic analysis of NB4-R1 (e) and NB4-R2 cells (f) treated with the indicated drugs for 72 hours. Similar results were obtained in three independent experiments. Differentiation was also evaluated by NBT reduction assay (g) and flow cytometric analysis of CD11b expression in NB4-R1 and NB4-R2 cells (h) with the indicated treatment for 72 hours. Each value represented the mean \pm SD of three independent measurements. ***, $P < 0.001$ as compared with DMSO. The representative histogram of flow cytometric analysis of CD11b expression in NB4-R1 (i) and NB4-R2 cells (j) with the indicated treatment for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

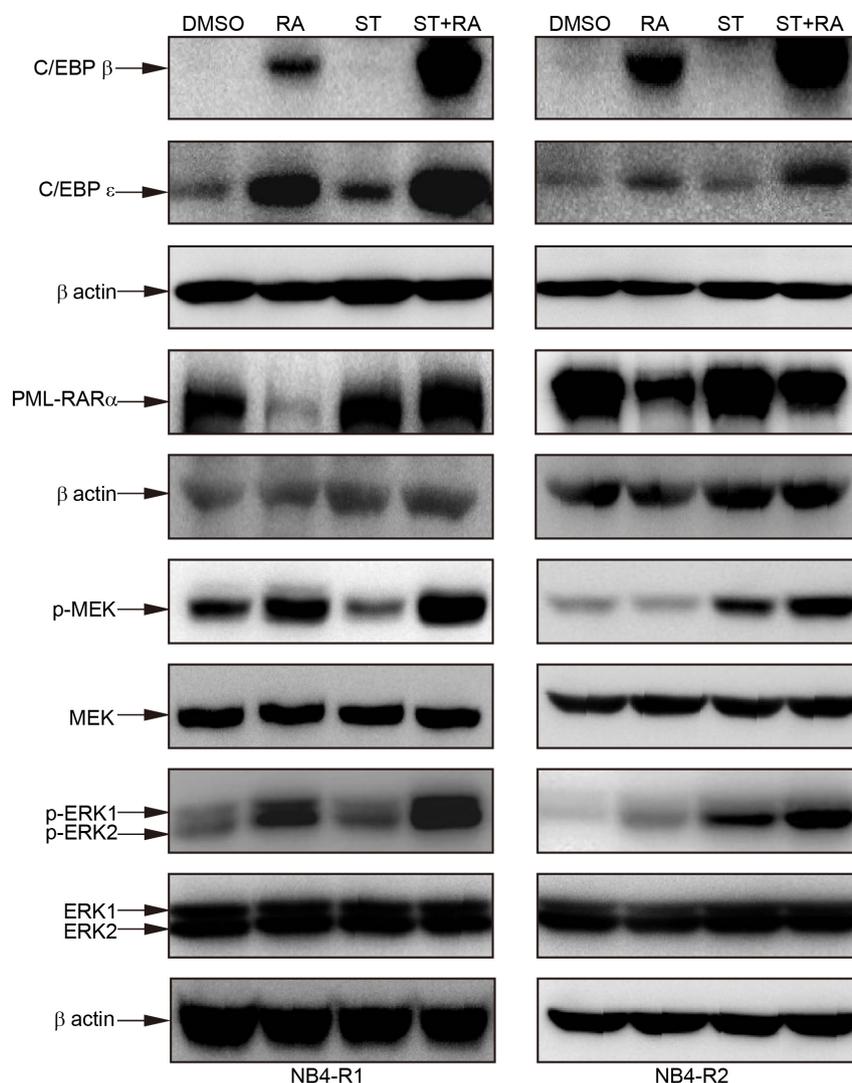


Figure 2 | Combined staurosporine and ATRA modulated the protein level of C/EBP β , C/EBP ϵ , PML-RAR α as well as the phosphorylation of MEK and ERK. Cells were treated with 2 nM staurosporine(ST), 1 μ M ATRA(RA) alone and the combined treatment (ST + RA) for 24 hours. The protein level of C/EBP β and C/EBP ϵ was measured by Western blot analysis using 20 μ g protein, while 50 μ g protein was loaded for Western blot analysis of the phosphorylation of MEK and ERK1/2. The same membrane incubated with the phosphorylated Erk1/2 or MEK1/2 was stripped and followed by detection of MEK and ERK1/2. For Western blot analysis of PML-RAR α , cells were treated with the indicated drugs for 72 hours and 20 μ g protein was loaded. Since different amount of protein and diverse time points for collecting protein were used, each has the expression of β -actin as internal control. Similar results were obtained in three independent experiments. The full size blots were shown in the Supplemental Fig. S1 online.

compared with ST + RA, $23.0 \pm 2.1\%$ vs $73.9 \pm 6.6\%$, $\chi^2 = 5186.60$, $P < 0.001$ in NB4-R1 cells and $21.9 \pm 2.6\%$ vs $75.1 \pm 2.3\%$, $\chi^2 = 5665.58$, $P < 0.001$ in NB4-R2 cells, Figure 3e–3g). In addition, in the presence of U0126, the combined treatment-enhanced C/EBP β and C/EBP ϵ protein level were remarkably suppressed in both cell lines (Figure 4a and 4b). These results highlighted a major role of MEK/ERK signal pathway in the differentiation-inducing effect of ATRA and staurosporine in ATRA resistant-APL cells.

Discussion

In the present work, we demonstrated that the combination of staurosporine and ATRA could overcome granulocytic differentiation block in retinoid resistant APL cell lines. Staurosporine has been proven to be not only an effective apoptosis inducing agent but also a potent differentiation inducer in a variety of tumor cell lines^{42–47}. To our knowledge, it is the first time to show that staurosporine can restore ATRA sensitivity in retinoid resistant APL cell lines. However, the effect of the combination on primary leukemia cells from retinoid resistant APL patients needs further investigation.

For the molecular mechanisms of the combination-induced differentiation, we focused on certain proteins or signaling pathways involving in granulocytic differentiation rather than PKC. C/EBPs are a family of transcription factors which regulate cell proliferation and differentiation. Among them, C/EBP α , C/EBP β and C/EBP ϵ are critical mediators of granulopoiesis. C/EBP α is required for the proceeding from myeloblast to promyelocyte while C/EBP β and C/EBP ϵ play central roles in terminal differentiation of granulocytes⁴⁸. Moreover, C/EBP β and C/EBP ϵ were demonstrated to be required for ATRA-mediated differentiation in APL cells^{49,50}. In addition, C/EBP ϵ alone could suppress the leukemia phenotype of APL and imitate the differentiation-induction effect of ATRA in APL mouse model⁵⁰. Thus, C/EBP β and C/EBP ϵ are not only the crucial regulators of granulocyte development but also the potential therapy target for leukemia. In this work, compared with any single treatment, the combination of staurosporine and ATRA remarkably increased the protein levels of both C/EBP β and C/EBP ϵ in NB4-R1 and NB4-R2 cells. Though any single treatment to some extent did up-regulate C/EBP β or C/EBP ϵ , we speculated that such increase might not be

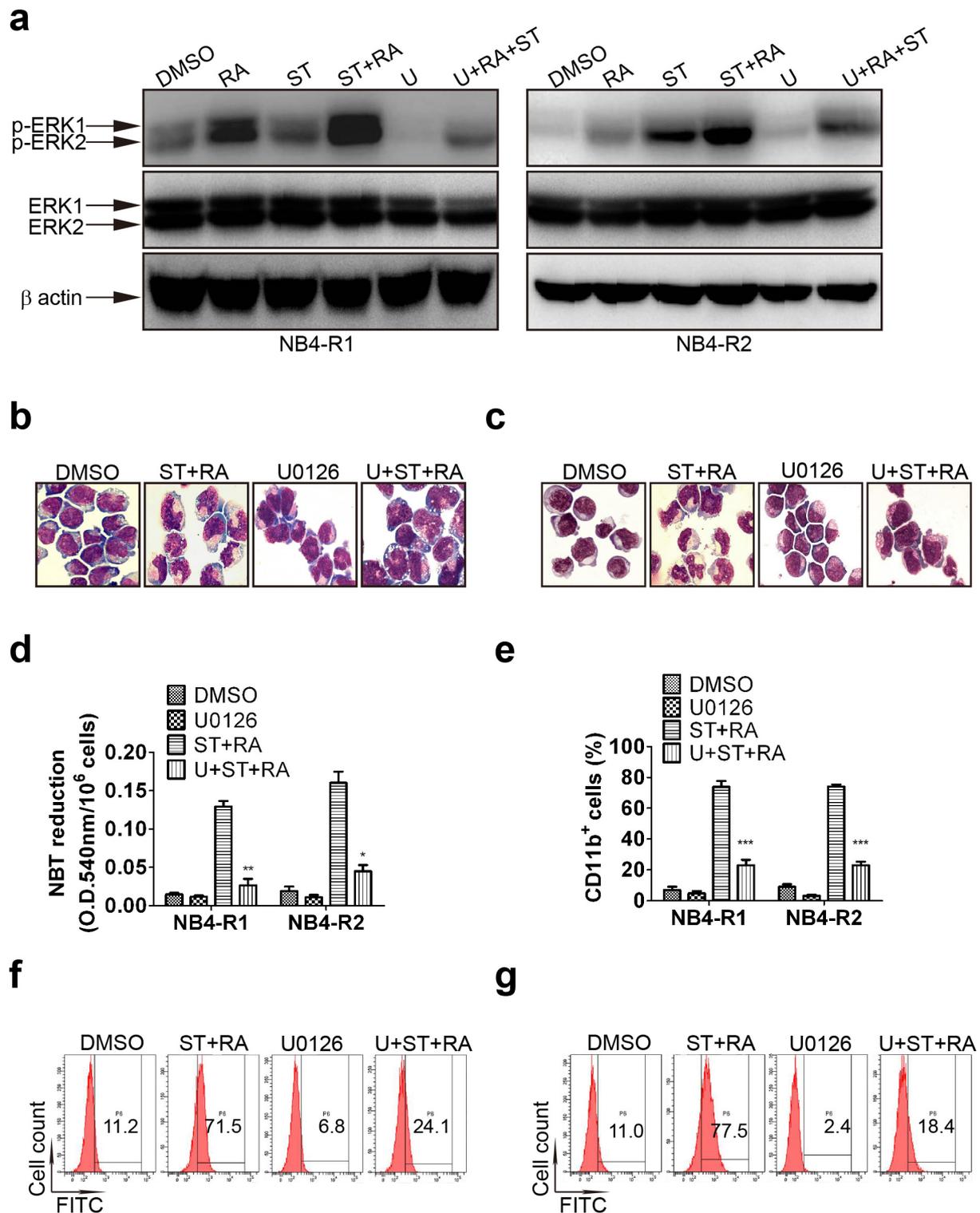


Figure 3 | Inhibition of MEK activation blocked the combined treatment-induced differentiation. Cells were exposed to 1 μ M U0126 for 1 hour prior to other treatment. The attenuation of MEK activation by U0126 (U) was measured by Western blot analysis of phosphorylated ERK1/2 in NB4-R1 and NB4-R2 cells with indicated treatments for 24 hours (a). Similar results were obtained in three independent experiments. The full size blots were shown in the Supplemental Fig. S2 online. Inhibitory effect of U0126 on morphologic changes in NB4-R1 (b) and NB4-R2 cells (c) incubated with the indicated drugs for 72 hours. One representative experiment among three independent assays was shown. The inhibitory effect of U0126 on differentiation was also confirmed by NBT reduction assay (d) and flow cytometric analysis of CD11b expression in NB4-R1 and NB4-R2 cells (e) with the indicated drugs for 72 hours. Each value represented the mean \pm SD of three independent measurements. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicated significant difference between U + ST + RA and ST + RA. The representative histograms of flow cytometric analysis of CD11b expression in NB4-R1 (f) and NB4-R2 cells (g) with the indicated drugs for 72 hours were also shown. The percentages of CD11b positive cells were shown in the corresponding panels.

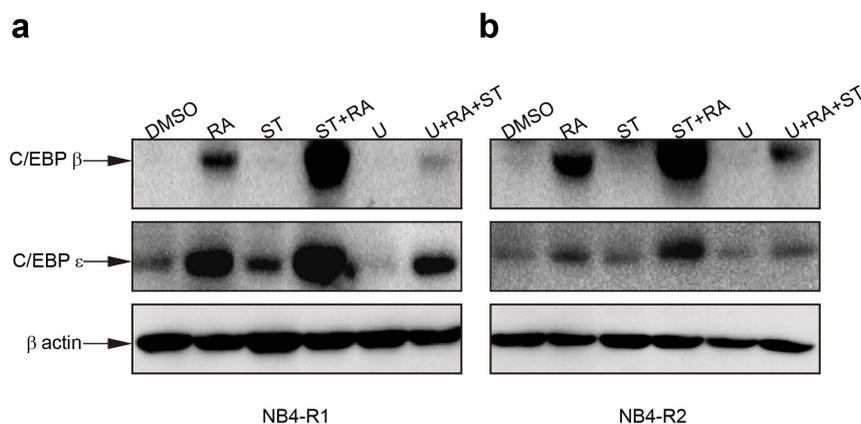


Figure 4 | Inhibition of MEK activation restored the protein levels of C/EBPs regulated by combined treatment. Cells were exposed to 1 $\mu\text{mol/L}$ U0126 for 1 hour prior to other treatments. The protein level of C/EBP β and C/EBP ϵ in NB4-R1 (a) and NB4-R2 (b) cells with the indicated drugs for 24 hours was determined by Western blot analysis. Expression of β -actin was assessed as internal control. Similar results were obtained in three independent experiments. The full size blots are shown in the Supplemental Fig. S3 online.

enough to trigger maturation but only combined treatment resulting in significant up-regulation of C/EBP β and C/EBP ϵ could speed up terminal differentiation.

It was well known that co-repressor release, co-activator recruitment, transcriptional activation of genes with the RAREs and PML-RAR α degradation were principal mechanisms of ATRA-induced differentiation of APL cells. However, it was indicated that pre-genomic activation of kinase signaling cascades also contributed to ATRA-induced differentiation^{51,52}. Our previous studies showed that MEK/ERK was activated during ATRA-induced granulocytic differentiation in APL cell line, NB4. Furthermore, inhibition of MEK activation suppressed almost all differentiation induced by ATRA, suggesting the significant role of MEK/ERK signaling pathway in ATRA-dependent NB4 cells granulocytic differentiation (unpublished data). Interestingly, the activation of MEK/ERK signaling pathway was also observed in the combination treatment. Moreover, attenuation of the MEK activation inhibited not only the differentiation but also the increased protein level of C/EBP ϵ and C/EBP β . Therefore, the protein level of C/EBP ϵ and C/EBP β was regulated by MEK/ERK signaling pathway and the later was required for the combination-induced differentiation. Accumulating evidence showed that MEK/ERK signaling pathway could promote C/EBP β expression and modify its activity^{53–55}. Moreover, in ATRA-treated APL cells, as the result of increasing protein level of C/EBP β , enhanced C/EBP β binding activity preceded that of C/EBP ϵ and C/EBP β itself could induce the expression of C/EBP ϵ ⁴⁹. Hence, there might be MEK-C/EBP β -C/EBP ϵ cascade in the combination-induced differentiation.

PML-RAR α fusion protein is believed to contribute to the pathogenesis of APL in a dominant negative fashion, disrupting the normal physiologic function of PML and repressing transcription by RARs. Either As₂O₃ or ATRA is regarded as one of the most successful examples of tumor targeted therapy since they both degraded APL pathogenic protein, PML-RAR α . However, in this work, though staurosporine inhibited ATRA-mediated PML-RAR α degradation, the combination of staurosporine and ATRA still synergistically induced differentiation. It meant that the combination could induce differentiation independent of PML-RAR α degradation, suggesting that the degradation of PML-RAR α might not be necessary for APL therapy. In consistent with our observation, other agents such as oridonin, G-CSF, STI571, pharicin B and TNF could cooperate with ATRA to induce differentiation in ATRA-resistant APL cell lines without any effect on PML-RAR α ^{26–29,56}. The increased protein level of C/EBP ϵ and C/EBP β by ATRA treatment was confirmed to depend on PML-RAR α expression^{49,50}. Therefore, it remains to be

further explored whether staurosporine prevented PML-RAR α degradation contributes to the enhanced protein level of C/EBP ϵ and C/EBP β as well as the combination-induced differentiation.

In conclusion, the combination of staurosporine and ATRA synergized to trigger differentiation in ATRA resistant APL cell lines by MEK/ERK signaling pathway. It remains to investigate whether such combination could induce differentiation in primary leukemia cells from retinoid resistant APL patients. The exact molecular mechanisms of how staurosporine overcomes ATRA resistance as well as more detailed mechanisms of the combination-induced differentiation need to be further elucidated.

Methods

Reagents. ATRA was purchased from Sigma-Aldrich (St Louis, MO) while both staurosporine and U0126 were obtained from EMD Chemicals (San Diego, CA). They were all dissolved by dimethylsilfoxide (DMSO) as a stock solution at 1 mM, 2 μM and 1 mM respectively.

Cell culture, cell viability and cell proliferation. The ATRA resistant cell lines, NB4-R1 and NB4-R2 cells were obtained from Dr Michel Lanotte (Hopital Saint Louis, Paris, France)⁵⁷ and were cultured in RPMI-1640, supplemented with 10% fetal calf serum (Thermo Fisher Scientific Inc, Waltham, MA) in a humidified atmosphere of 95% air/5% CO₂ at 37°C. To avoid possible effects of cell density on cell growth and survival, cells were maintained at less than 5×10^5 cells/ml. Cell viability was assayed by trypan-blue exclusion. Actual viable cell numbers were calculated by multiplying diluted times with counted viable cell numbers. Proliferation inhibition rate (%) = (control group actual viable cell numbers - experimental group actual viable cell numbers) / control group actual viable cell numbers $\times 100\%$.

Cell differentiation assays. Cell maturation was evaluated by cellular morphology, NBT reduction assay and the content of cell surface differentiation-related antigen CD11b. Morphology was determined with May-Grunwald-Giemsa's staining of cells centrifuged onto slides by cytopsin (Shandon, Runcon, UK; 500 r.p.m., 5 min) and viewed at $\times 1,000$ magnification. NBT reduction was performed as previously described⁵⁸. Briefly, 1×10^6 cells were collected and incubated with 1 mg/ml NBT (Sigma-Aldrich) solution containing 10 μM phorbol 12-myristate 13-acetate (Sigma-Aldrich) at 37°C for 1 hour. Cells were lysed by 10% sodium dodecyl sulfate (SDS) and 0.04 N hydrochloric acid (HCl). The absorbance at O.D 540 nm was detected by spectrophotometer (Beckman Coulter, Brea, CA). The expression of cell surface differentiation-related antigen CD11b was determined on flow cytometry (EPICS XL, Coulter, Hialeah, FL). Fluorochrome-labeled anti-human CD11b/FITC antibody was purchased from Coulter-Immunotech (Marseilles, France).

Annexin-V analysis. Annexin-V assay was performed according to instructions provided in the Annexin V-PI Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA). Briefly, 5×10^5 cells were harvested and washed with binding buffer provided in the kit. Then, cells were incubated with 5 μl annexin-V and 10 μl propidium iodide (PI) at room temperature in the dark for 15 min. Fluorescent intensities was determined on flow cytometry (Coulter).

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer (Sigma-Aldrich). Cell lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernants were collected and quantified by Bio-Rad Dc



protein assay (Bio-Rad Laboratories, Hercules, CA). 20 or 50 μ g protein extracts were loaded on 8% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred to polyvinylidene difluoride membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5% nonfat milk in PBS, the membranes were probed with antibodies against RAR α (Santa Cruz Biotech, Santa Cruz, CA), C/EBP β (Santa Cruz Biotech), C/EBP ϵ (Santa Cruz Biotech), anti- β -actin (Santa Cruz Biotech), phosphor-p44/Erk1/2 (Thr202/Try 204) (Cell Signaling Technology, Beverly, MA) and phosphor-MEK1/2 (Ser217/Try 221) (Cell Signaling Technology). Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Ltd). Immunocomplexes were visualized by chemiluminescence kit (GE Healthcare UK Ltd) according to the manufacturer's instruction. To detect Erk1/2 and MEK1/2, the same membrane incubated with the phosphorylated Erk1/2 or MEK1/2 was stripped with stripping buffer (2% SDS, 100 mM β -mercaptoethanol, 50 mM Tris, pH6.8) followed by blocking and probing with anti-Erk1/2 (Cell Signaling Technology) or anti-MEK1/2 (Cell Signaling Technology).

Statistical analysis. For NBT reduction, two-tailed paired Student's *t* test was used, *n* value was 3. The flow cytometric analysis of CD11b was analyzed by chi-square test, *n* value was 20,000.

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Author contributions

D.Z.G. and Y.S. carried out the experiments. D.Z.G. prepared all the figures. X.C. designed the study and wrote the manuscript. All authors reviewed the manuscript.

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