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Original Article

Enzastaurin enhances ATRA-induced differentiation of acute myeloid leukemia cells

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Abstract: All-trans retinoic acid (ATRA) is considered to be the sole clinically-useful differentiating agent in the treatment of acute myeloid leukemia (AML). However, ATRA has been effective only in acute promyelocytic leukemia (APL) but not other subtypes of AML. Therefore, discovering strategies to sensitize cells to ATRA may lead to the development of ATRA-based treatments in non-APL AML patients. In the present study, a clinically-achievable concentration of enzastaurin enhanced ATRA-induced differentiation in AML cell lines, HL-60 and U937 as well as non-APL AML primary cells. Furthermore, it also restored ATRA sensitivity in ATRA-resistant cell line, HL-60Res. Mechanistically, in all these cell lines, enzastaurin-ATRA (enz-ATRA) co-treatment enhanced the protein levels of PU.1, CCAAT/enhancer-binding protein β (C/EBPβ) and C/EBPε. The activity of protein kinase C β (PKCβ) was suppressed by enz-ATRA treatment in HL-60 and HL-60Res cells. However, another PKCβ-selective inhibitor mimicked the cellular and molecular effects of enzastaurin only in HL-60 cells. Furthermore, in U937 cells, enz-ATRA activated MEK and ERK, and a MEK-specific inhibitor suppressed enz-ATRA-triggered differentiation and reduced the protein levels of PU.1, C/EBPβ and C/EBPε. Enz-ATRA activated Akt in HL-60 and HL-60Res cells. However, an Akt inhibitor blocked enz-ATRA-triggered differentiation and restored the protein levels of PU.1, C/EBPβ and C/EBPε only in HL-60Res cells. Therefore, PKCβ inhibition, MEK/ERK and Akt activation were involved in enz-ATRA-induced differentiation in HL-60, U937 and HL-60Res cells, respectively, via modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Taken together, our findings may help to guide novel therapeutic strategies for AML patients.

Keywords: Acute myeloid leukemia, all-trans retinoic acid, differentiation, enzastaurin

Introduction

Acute myeloid leukemia (AML) accounts for 80% of adult acute leukemia [1]. The median age at diagnosis is approximately 70 years with five-year survival rate of only 10% for patients above the age of 60 years while 40% for younger patients (18-60 years) [1]. Due to the improvement of lifespan of the general population, AML is predicted to increase 38% in elder patients by 2031. Over the last four decades, cytarabine/anthracycline-based chemotherapy has been the main therapeutic strategy for AML patients resulting in a remission rate of 60-85% for patients younger than 60 years of age and 40-60% for elder patients [1]. However, most patients relapse and become resistant to treatment. As mentioned above, survival is worse for elderly patients who neither can tolerate intensive treatment nor are suitable for stem cell transplantation. Therefore, there is a pressing need to develop new therapeutic strategies for AML patients. Over the last couple of years, some new genetic driver mutations in AML have been identified and several mutation-targeted agents with promising results in clinical trials have been developed [2]. However, only a small portion of AML patients can benefit from these novel treatment strategies. Thus, the development of other effective anti-AML therapies is still required.

Differentiation therapy, which clears tumor bulk by terminal maturation with relatively less severe side effects, may be an alternative to chemotherapy in this circumstance. All-trans

retinoic acid (ATRA), the active metabolite of vitamin A, has been successfully applied in the treatment of acute promyelocytic leukemia (APL) by differentiation induction [3]. However, due to the complicated physiopathology of non-APL AML, a clinical trial of ATRA in AML had disappointing results [4]. Since ATRA is a master regulator of myeloid cell differentiation, research strategies to extend the efficacy of ATRA-based therapy to non-APL AML is ongoing. A mutation *in nucleophosmin1 (NPM1)* without *FLT3 internal tandem duplications (FLT3-ITDs),* R132H mutation in *isocitrate dehydrogenase 1 (IDH1)* or overexpression of *ecotropic viral integration site 1 (EVI-1)* has been demonstrated to increase the response of non-APL AML cells to ATRA [5-7], suggesting that AML patients with certain genetic alterations might benefit from ATRA-based therapy. Specifically, a combination of ATRA with chemotherapy, epigenetic modifiers or arsenic trioxide may be a rational approach to some AML patients [8]. Alternative strategies to increase the expression or the activity of retinoic acid receptor α (RARα) or inhibit its degradation have shown to restore the sensitivity of AML cells to ATRA *in vitro* or *in vivo* [8].

The MEK/ERK pathway is required for myeloid differentiation induced by certain cytokines and ATRA-triggered differentiation in HL-60 and APL cells [9-12]. Except for limited studies demonstrating that Src inhibitors can promote ATRA-induced differentiation in AML cells by MEK/ERK, the MEK/ERK pathway has rarely been used to improve ATRA sensitivity in AML cells [13, 14]. Since MEK/ERK is an important cytoplasmic pathway for myeloid differentiation, it may serve as a target for enhancing ATRA sensitivity in AML cells. Enzastaurin, a derivative of protein kinase C (PKC) pan-inhibitor staurosporine, has been designed to suppress the activation of PKCβ [15]. It has been proven to be safe and well tolerated in multiple clinical trials, and has shown promising anticancer activity [15]. Moreover, it can also reverse ATRA resistance and synergize with ATRA to induce differentiation in ATRA-resistant APL cells via MEK/ERK [16]. However, whether enzastaurin can promote ATRA-induced differentiation in AML cells has not yet been investigated.

In this study, non-APL AML cell lines HL-60, U937 and the ATRA-resistant HL-60 cell line, HL-60Res were used as *in vitro* models. Each of these cell lines do not have an *NPM1* mutation, which is associated with increased responsiveness to ATRA [17, 18]. We found that a clinical-achievable concentration of enzastaurin enhanced ATRA-induced differentiation in HL-60, U937 and non-APL AML primary cells while it also reversed ATRA resistance in HL-60Res cells. Mechanistically, different pathways were involved in. PKCβ inhibition, MEK/ERK and AKT regulated the combination of enzastaurin and ATRA (enz-ATRA) induced differentiation in HL-60, U937 and HL-60Res cells, respectively. PU.1, CCAAT/enhancer-binding protein β (C/EBPβ) and C/EBPε were the downstream molecules of these signaling pathways.

Material and methods

Reagents

ATRA was obtained from Sigma-Aldrich (St Louis, MO, USA). Enzastaurin, trametinib and Ly294002 were purchased from Selleckchem Chemicals (Houston, TX, USA). A PKCβ inhibitor was obtained from Merck (Darmstadt, Germany). All reagents were dissolved in dimethyl sulfoxide (DMSO).

Primary cells and cell culture

Bone marrow samples were collected at the time of diagnosis at the Department of Hematology of Ruijin Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by the Medical Science Ethic committee in the School of Medicine at Shanghai Jiao Tong University. Mononuclear cells were isolated by density-gradient centrifugation via Ficoll-Paque Plus (GE healthcare Biosciences, Uppsala, Sweden) and were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (GE healthcare Bio-sciences) supplemented with 20% fetal bovine serum (GE healthcare bio-sciences), 10 ng/mL recombinant human interleukin-3 (rhIL-3), 10 ng/mL rhIL-6 and 50 ng/mL recombinant human stem cell factor (rh SCF) (PeproTech Inc China, Suzhou, Jiangsu, China).

HL-60 and HL-60Res cells were cultured in IMDM supplemented with 20% fetal bovine serum while U937 cells were cultured in RPMI-

1640 medium (GE healthcare bio-sciences), supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Annexin-V analysis

According to instructions provided in the Annexin V-7AAD Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA, USA), 5×10^5 cells were harvested and washed with binding buffer. Subsequently, cells were incubated with 5 μL 7-Amino-Actinomycin and 5 μL annexin-V in the dark at room temperature for 15 min. Fluorescent intensities were evaluated by flow cytometry (EPICS XL, Coulter, Hialeah, FL, USA).

Cell differentiation assays

Cell maturation was determined by cellular morphology and the content of cell-surface differentiation-related antigen CD11b. Morphology was evaluated via May-Grunwald-Giemsa's staining and observed at 1000× magnification. The expression of cell surface differentiation-related antigen CD11b (Coulter, Marseilles, France) was determined by flow cytometry (EPICS XL).

Western blot analysis

After lysed with RIPA buffer (Sigma-Aldrich) and centrifuged at 13,000 rpm for 10 min at 4°C, supernatants were collected and quantified via Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Then, 20 or 50 μg protein extracts were loaded onto 8% SDS-polyacrylamide gels, subjected to electrophoresis, and transferred to polyvinylidene-difluoride membranes (GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5% nonfat milk or BSA in PBS, the membranes were incubated with the following primary antibodies: C/EBPβ, C/EBPε, PU.1 from Santa Cruz Biotech (Santa Cruz, CA, USA); phospho-p44/42 Erk1/2 (Thr202/Try204), phospho-MEK1/2 (Ser218/222), phospho-PKC (pan) (βII Ser660), phospho-PKCα/β II (Thr638/641), phospho-Akt (Ser473) and phospho-Akt (Thr308) from Cell Signaling Technology (Beverly, MA, USA); GAPDH from Proteintech (Rosemont, IL, USA). Then membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Ltd). Immunocomplexes were visualized with chemiluminescence kit (GE Healthcare UK Ltd). To detect Erk1/2, MEK1/2, PKCβ and AKT, the same membrane incubated with accordingly phosphorylated antibody was stripped with stripping buffer (2% SDS, 100 mM of beta-mercaptoethanol, 50 mM of Tris, pH6.8), followed by blocking and probing with anti-Erk1/2 (Cell Signaling Technology), anti-MEK1/2 (Cell Signaling Technology), anti-PKCβ (Santa Cruz Biotech) or anti-AKT (Cell Signaling Technology), respectively.

Statistical analysis

Chi-square tests (n = 20,000) were used to analyze flow-cytometric analysis of CD11b. $P<0.05$ was taken to indicate statistical significance.

Results

Enzastaurin enhances ATRA-induced differentiation in HL-60 U937 and patient-derived AML cells while also reversing ATRA resistance in HL-60Res cells

Since 2 μM has been demonstrated to be a clinically-achievable concentration of enzastaurin [19], this concentration was used as the maximum concentration of enzastaurin in all cell lines in the present study. We found that 2 μM of enzastaurin alone or in combination with ATRA inhibited cell growth in HL-60 and U937 cells (**Figure 1A**, **1C**), while proliferation was not affected with any treatment in HL-60Res cells (**Figure 1B**). The cell viability was maintained above 95% with any treatment in all cell lines (**Figure 1D-F**). Meanwhile, the content of Annexin V^+ cells only increased slightly with some treatments in all cell lines (**Figure 1G-I**).

Morphologically, as illustrated in **Figure 2A**, all cell lines presented a characteristic morphology of primitive cells such as a round nucleus and a large nuclear/cytoplasmic ratio. With ATRA treatment for 3 d in HL-60 and U937 cells, some cells displayed decreased nuclear/cytoplasmic ratio with kidney-shape nuclei. However, there was no obvious change in HL-60Res cells with ATRA treatment for 10 d. More mature cells were presented in all cell lines with a combination of any concentration of enzastaurin and ATRA, especially with co-treatment of ATRA and 2 μM enzastaurin

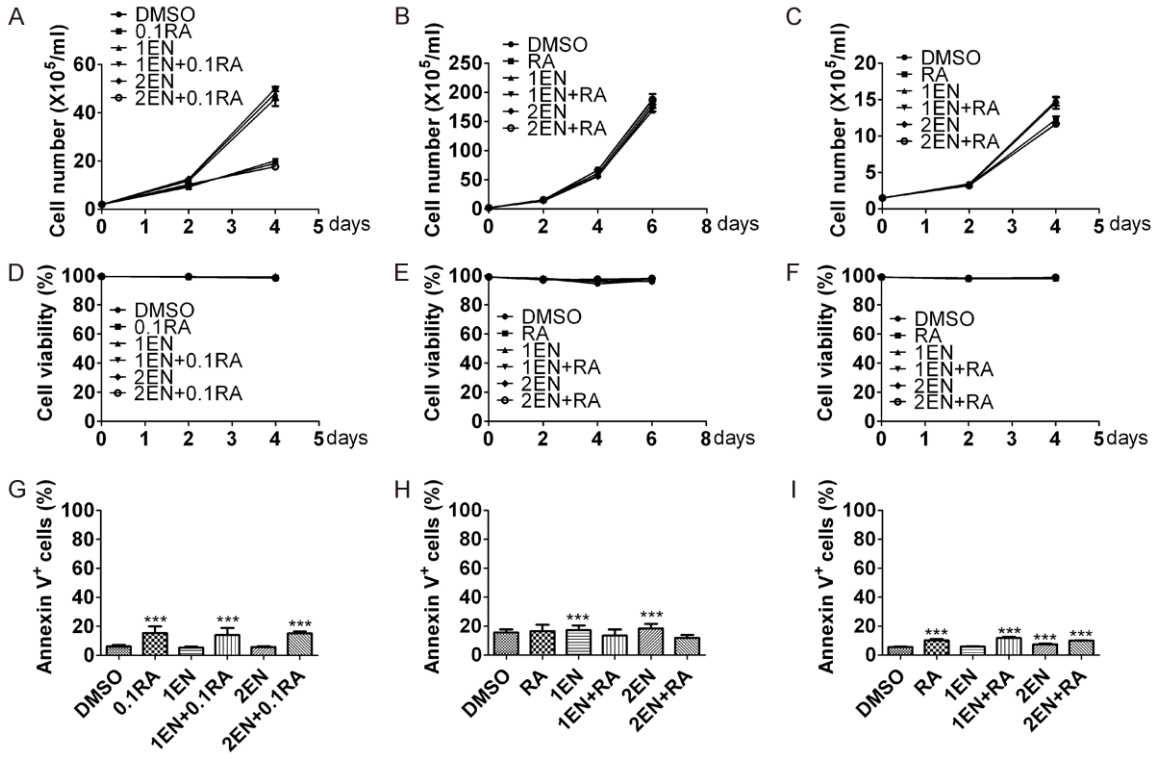


Figure 1. The effects of enz-ATRA treatment on cell growth and survival in HL-60, HL-60Res and U937 cells. U937 (right column) and HL-60Res (middle column) cells were treated with 1 μM of enzastaurin (1EN), 2 μM of enzastaurin (2EN), 1 μM of ATRA (RA) or an enz-ATRA combination (EN+RA) for the indicated days. HL-60 cells (left column) were treated with 1 μM of enzastaurin (1EN), 2 μM of enzastaurin (2EN), 0.1 μM of ATRA (0.1RA) or enz-ATRA co-treatment (EN+0.1RA) for 4 d. Representative experiments of cell growth (A-C) and cell viability (D-F) are shown. Each value represents the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. Annexin-V assays of HL-60 (G), U937 (I) and HL-60Res (H) cells treated with enzastaurin and/or ATRA for 4, 3 and 6 d, respectively. Each value represents the mean ± SD of three independent measurements. ***P<0.001 versus DMSO-treated cells.

(**Figure 2A**). Consistent with the morphological results, a synergistic effect of enzastaurin and ATRA on the content of $CD11b^+$ cells was also observed in a dose-dependent manner in all cell lines (**Figure 2B-E**). Therefore, enzastaurin enhanced ATRA-induced differentiation in HL-60 and U937 cells while restoring ATRA sensitivity in HL-60Res cells. For non-APL AML primary cells, five out of nine samples were AML-M4 and AML-M5 (**Table 1**). In four out of these five samples, enzastaurin enhanced ATRA-induced differentiation as assayed by morphology and the content of $CD11b^+$ cells (**Figure 3A**, **3B**). In samples diagnosed as AML-M1 (No.2), AML-M2 (No.8 and No.9), MDS transforming to AML (No.7) and AML-M5 with AML-ETO fusion gene and *c-kit* mutation (No.6), such effects were not observed (**Table 1**). One difference between these two groups was that four cases that were effective to enz-ATRA had partial response to ATRA, while five cases that were invalid to enz-ATRA had no response to ATRA (**Table 1**). Thus, enzastaurin enhanced ATRA-induced differentiation in some AML primary cells.

Enzastaurin enhances ATRA-induced differentiation in HL-60 cells via inhibition of PKCβ

To investigate the mechanisms of enz-ATRA treatment-triggered differentiation in these three cell lines, we used 2 μM enzastaurin in the following studies. Since enzastaurin has been designed to suppress the activation of PKCβ [15], we first studied the role of PKCβ in enz-ATRA-induced differentiation. Phosphorylation of Ser660 or Thr641 is essential for activation of PKCβ [20]. As shown in **Figure 4A**, compared with that of ATRA treatment, enz-ATRA treatment decreased the phosphoryl-

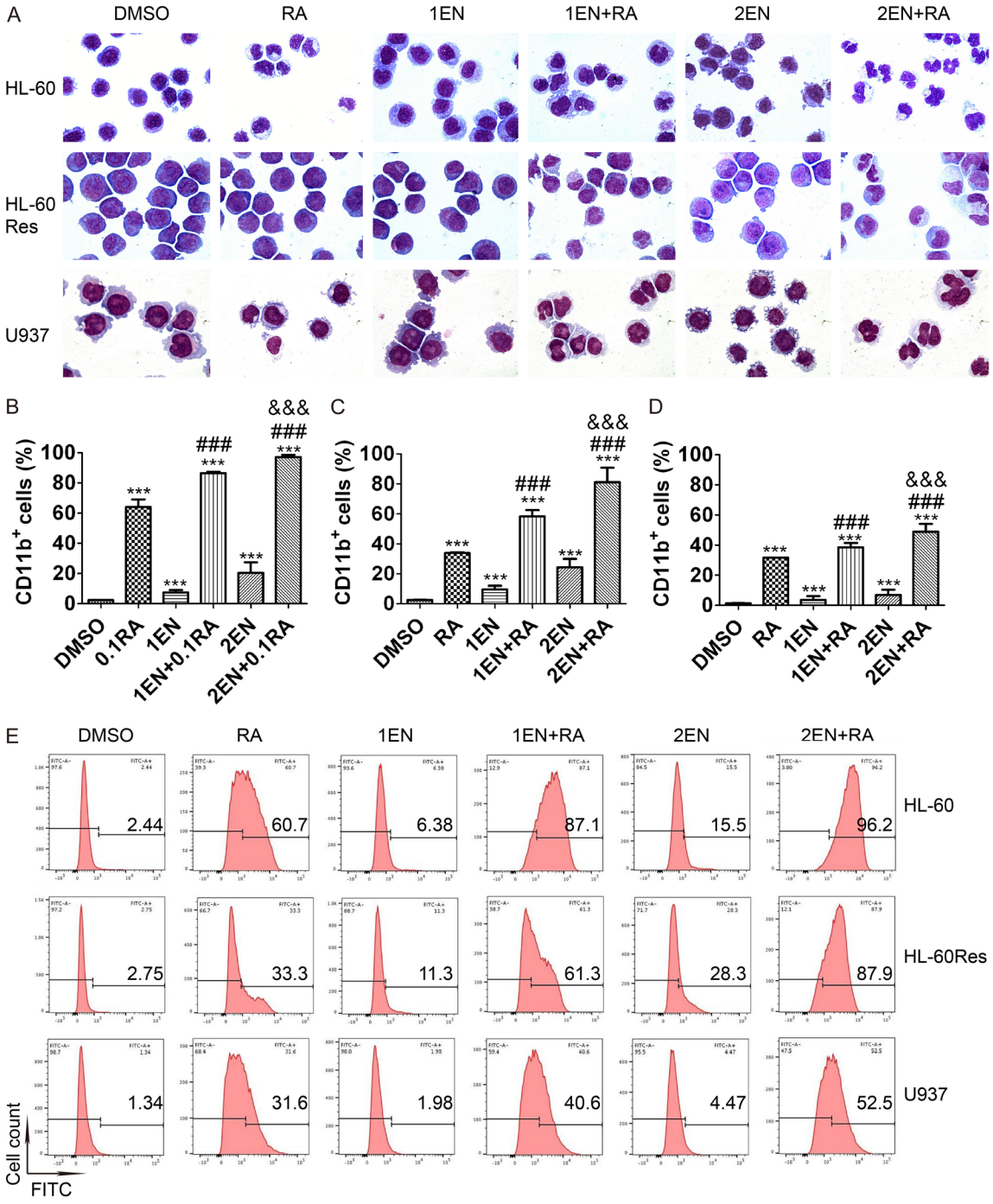


Figure 2. The effects of enz-ATRA treatment on cell differentiation in HL-60, HL-60Res and U937 cells. (A) HL-60 cells were treated with 1/2 μM of enzastaurin (1EN/2EN) and/or 0.1 μM of ATRA (RA) for 3 d. HL-60Res and U937 cells were treated with 1/2 μM of enzastaurin (1EN/2EN) and/or 1 μM of ATRA (RA) for 10 and 3 d, respectively. One representative morphological image of HL-60 (upper panel), HL-60Res (middle panel) and U937 (lower panel) cells are shown. Magnification is 1,000×. Similar results were obtained in three independent experiments. Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60 (B), HL-60Res (C) and U937 cells (D) with the indicated treatment for 4, 6 and 3 d, respectively. Each value represents the mean ± SD of three independent measurements. ***P<0.001, versus DMSO-treated cells. ###P<0.001, versus ATRA-treated cells. &&&P<0.001, as compared with 1EN+RA in U937 and HL-60Res cells or 1EN+0.1RA in HL-60 cells. (E) Representative histograms of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4, 6 and 3 d, respectively. The percentages of $CD11b^+$ cells are shown in the corresponding panels.

Table 1. Patients data and response to enzastaurin and/or ATRA

No.	Sex	Age	Karyotype	Gene mutation/fusion	Blast (%)	WBC ($\times 10^9$/L)	FAB classification	$CD11b^+$ cells (%)			
								DMSO	RA	EN	EN+RA
1	M	65	46,XY,t(6;11)(q27;q23)	MLL-AF6	85.5	9.17	AML-M4	9.53	33.4	33.9	56.7
2	M	55	46,XY	*C/EBPα* G141C mutation, *C/EBPα* P192_H193 inserts PP, *C/EBPα* A303_K313 duplication, C/*EBPα* Q305_R306 inserts HNVETQQKAKQ	88	288	AML-M1	0.6	3.8	0.2	5.5
3	M	57	46,XY	*FLT3*Y599_D600 inserts GSTGSSDNEYFYVDFREYEY	50	97.73	AML-M5	19.4	20.8	26.9	37.5
4	F	44	48~49,XX,t(10;11)(p12;q23),+21*3	MLL-AF10, *N-RAS* mutation	83	7.8	AML-M5a	3.33	24	9.87	33.4
5	M	76	45~47,XY,-7,+M1~M6	*NRAS* G12S mutation	73	74.71	AML-M4b	10.4	53	20.4	63.5
6	F	55	45,X,-X,t(8;21;12)(q22;q22p13)	AML1-ETO, *C-KIT* T380 duplication	86	14	AML-M5	3.2	3.9	9.3	9
7	M	51	46,XY	*DNMT3A* N-terminal catalytic domain mutation	ND	3.79	MDS transforming to AML	3	3.8	4.9	5.8
8	F	56	46,XX	*C/EBPα* K313 duplication	54	8.45	AML-M2a	2.2	1.9	7.2	8
9	F	38	46,XX	*C/EBPα* Q305P mutation	36.5	6.12	AML-M2	0.8	0.8	1.8	2.6

Mononuclear cells were isolated by density-gradient centrifugation and were maintained in IMDM supplemented with 20% fetal bovine serum, 10 ng/mL rhIL-3, 10 ng/mL rhIL-6 and 50 ng/mL rhSCF. The cells were treated with 2 μM enzastaurin (EN) and/or 1 μM ATRA (RA) for 4 d. CD11b-positive cells were calculated by flow cytometry. ND indicates not done.

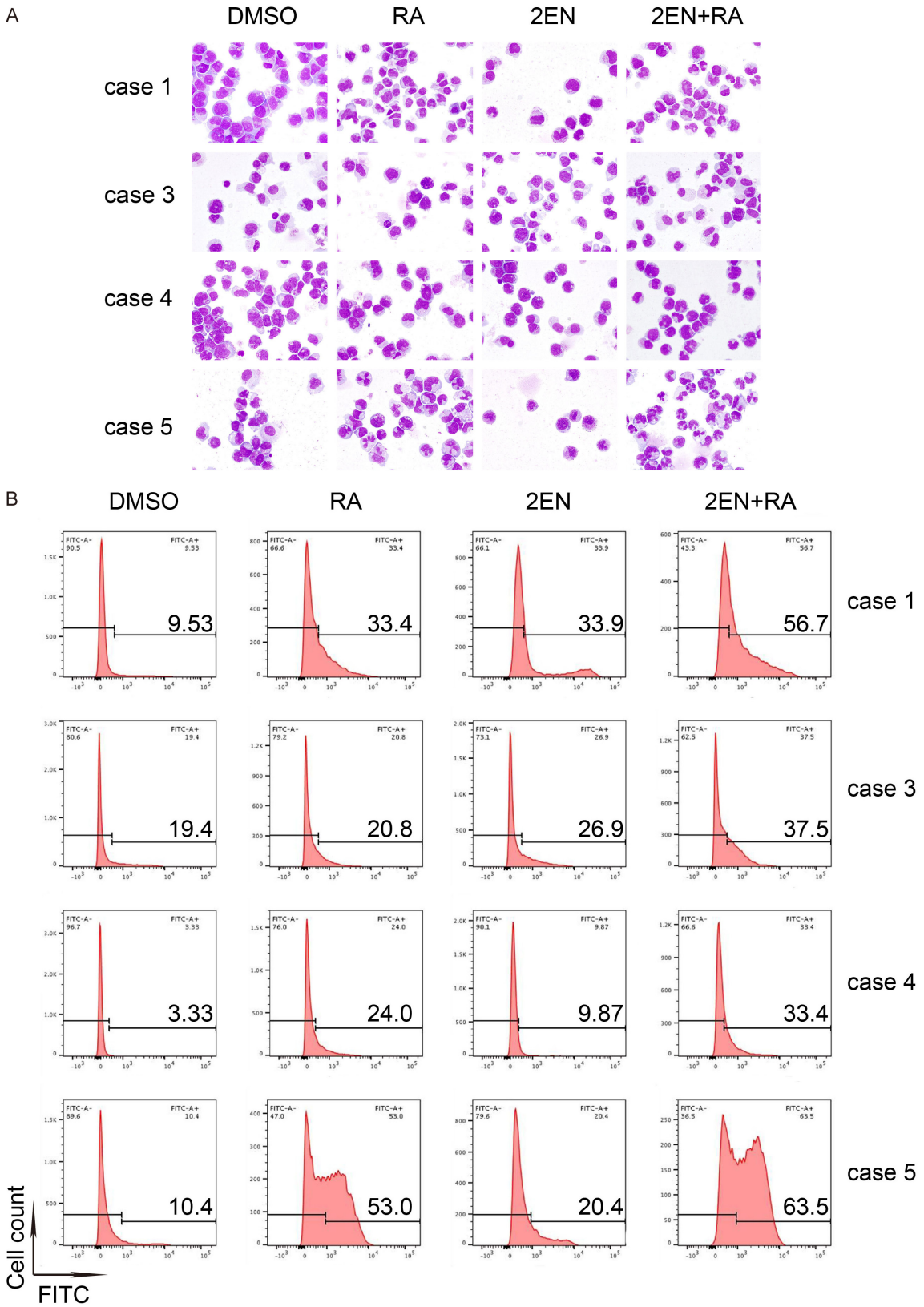
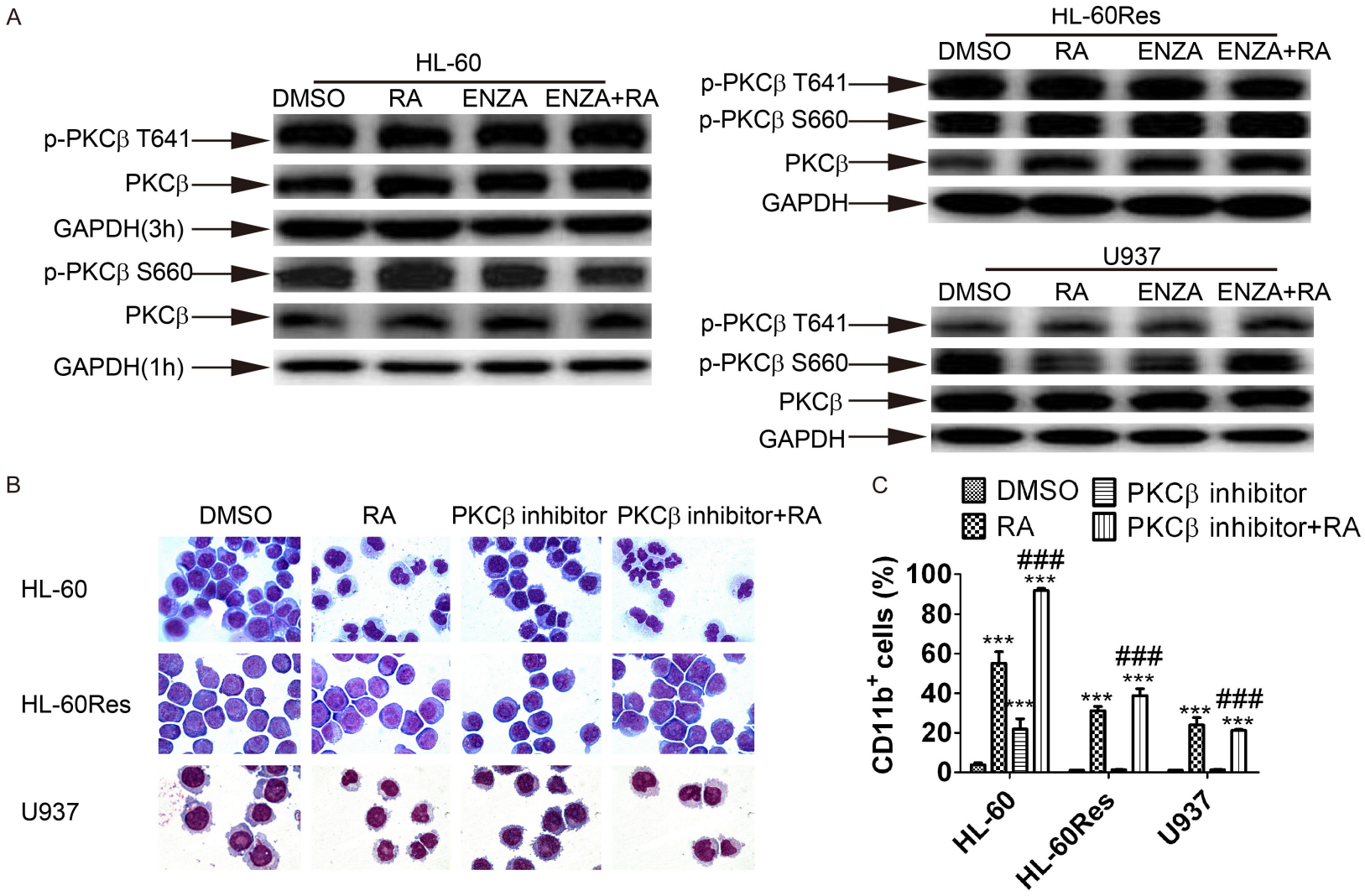


Figure 3. Enzastaurin enhances ATRA-induced differentiation in some primary cells from non-APL AML patients. Primary cells from patients were treated with 2 μM of enzastaurin (EN) and/or 1 μM of ATRA (RA) for 4 d. The morphology (A) and the histograms of flow-cytometric analysis of CD11b expression (B) are shown. Magnification of morphology is 1,000×. The percentages of $CD11b^+$ cells are shown in the corresponding panels.

ation of PKCβ S660 in HL-60 cells, while phosphorylation of PKCβ T641 was reduced in HL-60Res cells. However, in U937 cells, phosphorylation of PKCβ S660 or PKCβ T641 was not reduced with enz-ATRA treatment compared with that of ATRA treatment. Thus, enzastaurin inhibited PKCβ in HL-60 and HL-60Res cells. To confirm the role of PKCβ, another PKCβ inhibitor was combined with ATRA to examine whether it could mimic the effect of enzastaurin to augment ATRA-induced differentiation. Specifically, 500, 200 or 100 nM of PKCβ inhibitor was used in HL-60, HL-60Res or U937 cells, respectively, with no obvious effects on survival. Of note, the concentration of PKCβ inhibitor that was used was 5- to 25-fold higher than the IC50 to inhibit PKCβI and PKCβII, as indicated in the instructions of the assay kit. Similar to the results of enz-ATRA treatment, fully differentiated cells with lobed nuclei accompanied by a markedly-decreased nuclear/cytoplasmic ratio were presented in HL-60 cells following PKCβ-inhibitor/ATRA treatment for 4 d (**Figure 4B**). Moreover, compared with that of enz-ATRA treatment, the content of $CD11b^+$ cells was increased to a similar level in HL-60 cells with PKCβ-inhibitor/ATRA treatment (**Figure 4C**, **4D**). Similar to enz-ATRA treatment, compared with that of ATRA treatment, phosphorylation of PKCβ S660 was reduced with PKCβ inhibitor in HL-60 cells (**Figure 4E**). Thus, by suppression of PKCβ, this PKCβ inhibitor enhanced ATRA-triggered differentiation in HL-60 cells similar to the effects of enzastaurin. These results suggest that PKCβ inhibition might regulate enzastaurin-enhanced ATRA-triggered differentiation in HL-60 cells. However, this PKCβ inhibitor neither elevated ATRA-triggered differentiation in U937 cells nor restored ATRA sensitivity in HL-60Res cells, as evaluated by morphology (**Figure 4B**) and the content of $CD11b^+$ cells (**Figure 4C**, **4D**). Therefore, PKCβ may not be involved in enz-ATRA treatment-triggered differentiation in U937 and HL-60Res cells.

PKCβ inhibition and MEK/ERK activation are involved in enz-ATRA-induced differentiation in HL-60 and U937 cells, respectively by upregulation of the protein levels of C/EBPβ, C/EBPε and PU.1

To further elucidate the mechanisms of enz-ATRA treatment-triggered differentiation, we investigated several proteins and signaling pathways involved in ATRA-induced differentiation in HL-60 cells and granulocytes. As mentioned above, the MEK/ERK signaling pathway regulates certain cytokines-induced myeloid differentiation and ATRA-triggered granulocytic differentiation in APL cells and HL-60 cells [9-12]. C/EBPβ, C/EBPε and PU.1 are required for the maturation of myeloid lineages, as well as ATRA-induced differentiation in APL cells [21-24]. Moreover, by MEK/ERK modulating the protein levels of C/EBPβ, C/EBPε and PU.1, some medicines including enzastaurin synergize with ATRA to induce differentiation in ATRA-resistant APL cells [16, 25-27]. As shown in **Figure 5**, compared with those following ATRA treatment, the protein levels of C/EBPβ, C/EBPε and PU.1 were significantly increased via enz-ATRA treatment in all cell lines. Meanwhile, compared with those of ATRA treatment, PKCβ-inhibitor/ATRA treatment also augmented the protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60 cells. This suggested that enzastaurin promoted ATRA-upregulated protein levels of C/EBPβ, C/EBPε and PU.1 via PKC inhibition to enhance ATRA-induced differentiation in HL-60 cells. However, compared with those following ATRA treatment, the phosphorylation levels of MEK and ERK were enhanced only in U937 cells with enz-ATRA treatment. In HL-60 and HL-60Res cells, ATRA phosphorylated MEK and ERK, while enz-ATRA did not elevate their phosphorylation levels. Trametinib, a highly specific and potent MEK1/2 inhibitor [28], attenuated MEK activity in all cell lines, as determined by western blotting of phosphorylated ERK1/2 (**Figure 6A**). With trametinib pretreatment, fully-differentiated cells with lobed nuclei and a decreased nuclear/cytoplasmic ratio were replaced by primitive cells with round nuclei and a large nuclear/cytoplasmic ratio in U937 cells (**Figure 6B**). The content of $CD11b^+$ cells was also significantly suppressed by trametinib in U937 cells (**Figure 6C**, **6D**). Moreover, in the presence of trametinib, enz-ATRA-enhanced protein levels of C/EBPβ, C/EBPε and PU.1 were remarkably decreased in U937 cells (**Figure 6E**). Thus, enz-ATRA treatment induced differentiation in U937 cells via MEK/ERK modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Furthermore, trametinib slightly inhibited enz-ATRA treatment-triggered differentiation in HL-60 cells while unexpectedly augmenting enz-ATRA treatment-


A
HL-60
DMSO
RA
ENZA
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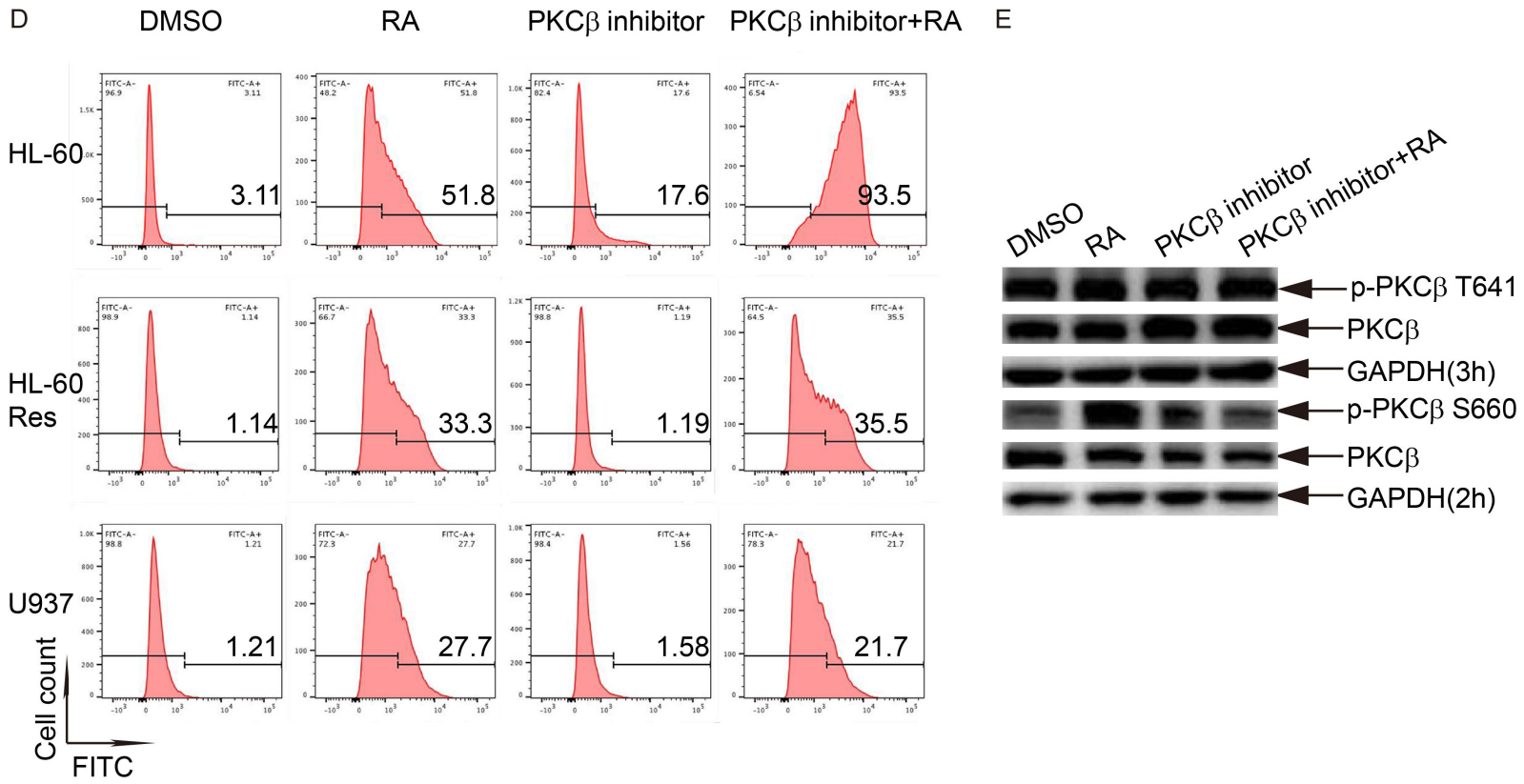


Figure 4. PKCβ-inhibition mediates enzastaurin-enhanced ATRA-triggered differentiation in HL-60 cells. A. HL-60 cells were treated with 2 μM of enzastaurin (EN) and/or 0.1 μM of ATRA (RA) for 1 or 3 h. HL-60Res and U937 cells were treated with 2 μM of enzastaurin (EN) and/or 1 μM of ATRA (RA) for 3 h. The activation of PKCβ was evaluated by western blotting analysis of phosphorylated PKCβ at serine 660 and threonine 641. The same membrane incubated with anti-phospho-PKCβ was stripped and followed by detection of PKCβ. Since diverse time points for collecting protein were used, each has the expression of GAPDH as an internal control. B. HL-60 cells were treated with 500 nM of PKCβ inhibitor and/or 0.1 μM of ATRA (RA) for 3 d. HL-60Res cells were treated with 200 nM of PKCβ inhibitor and/or 1 μM of ATRA (RA) for 10 d. U937 cells were treated with 100 nM of PKCβ inhibitor and/or 1 μM of ATRA (RA) for 3 d. Representative morphologies of HL-60, HL-60Res and U937 cells are shown. Magnification is 1,000×. Similar results were obtained in three independent experiments. C. Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4, 6 and 3 d, respectively. Each value represents the mean ± SD of three independent measurements. ***P<0.001, versus DMSO-treated cells. ###P<0.001, versus ATRA-treated cells. D. Representative histograms of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4, 6 and 3 d, respectively. The percentages of $CD11b^+$ cells are shown in the corresponding panels. E. HL-60 cells were treated with 500 nM of PKCβ inhibitor and/or 0.1 μM of ATRA (RA) for 2 or 3 h. Phosphorylation of PKCβ was measured by western blotting analysis. The same membrane incubated with anti-phospho-PKCβ was stripped and followed by detection of PKCβ. Since diverse time points for collecting protein were used, each has the expression of GAPDH as internal control.

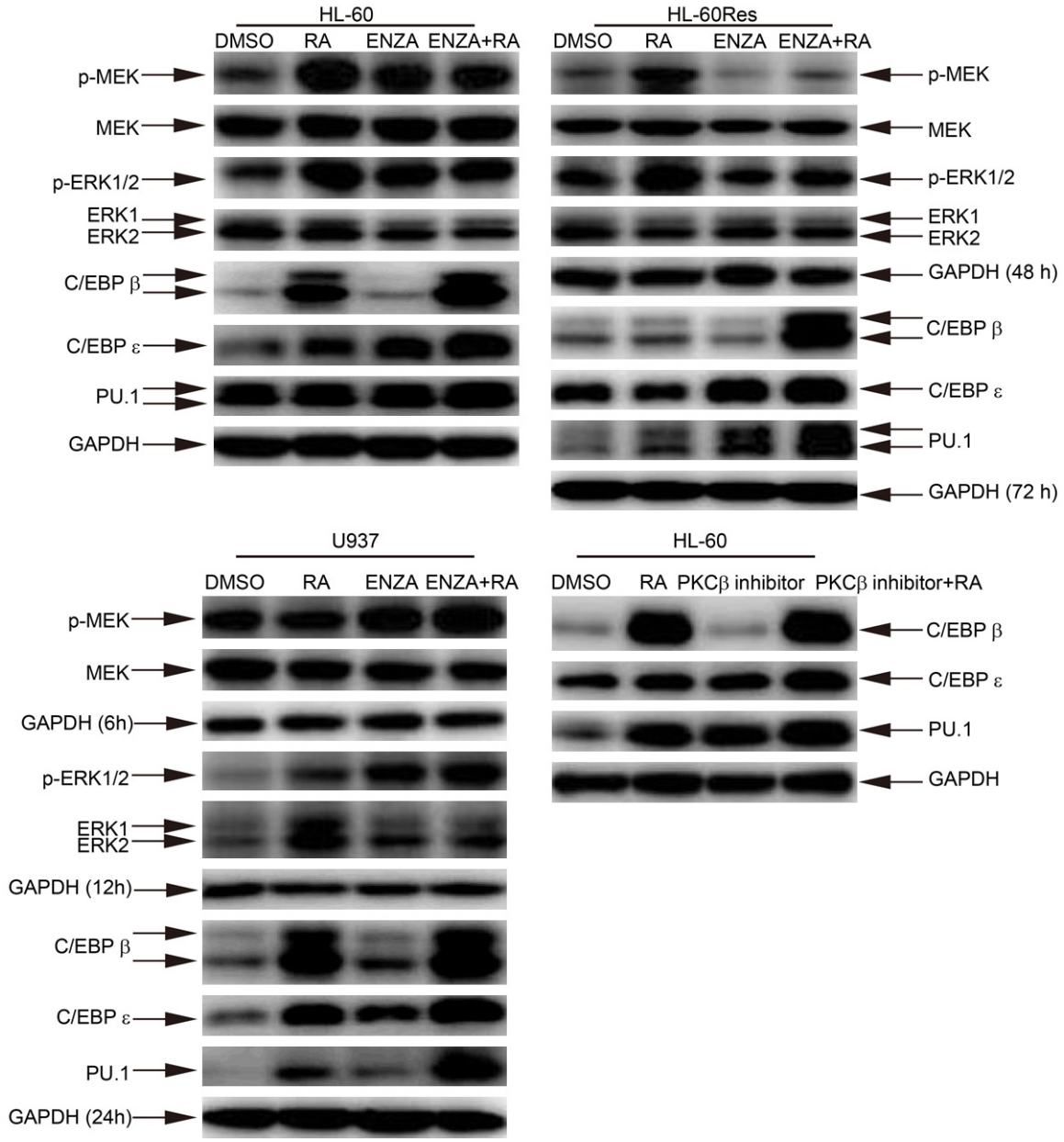
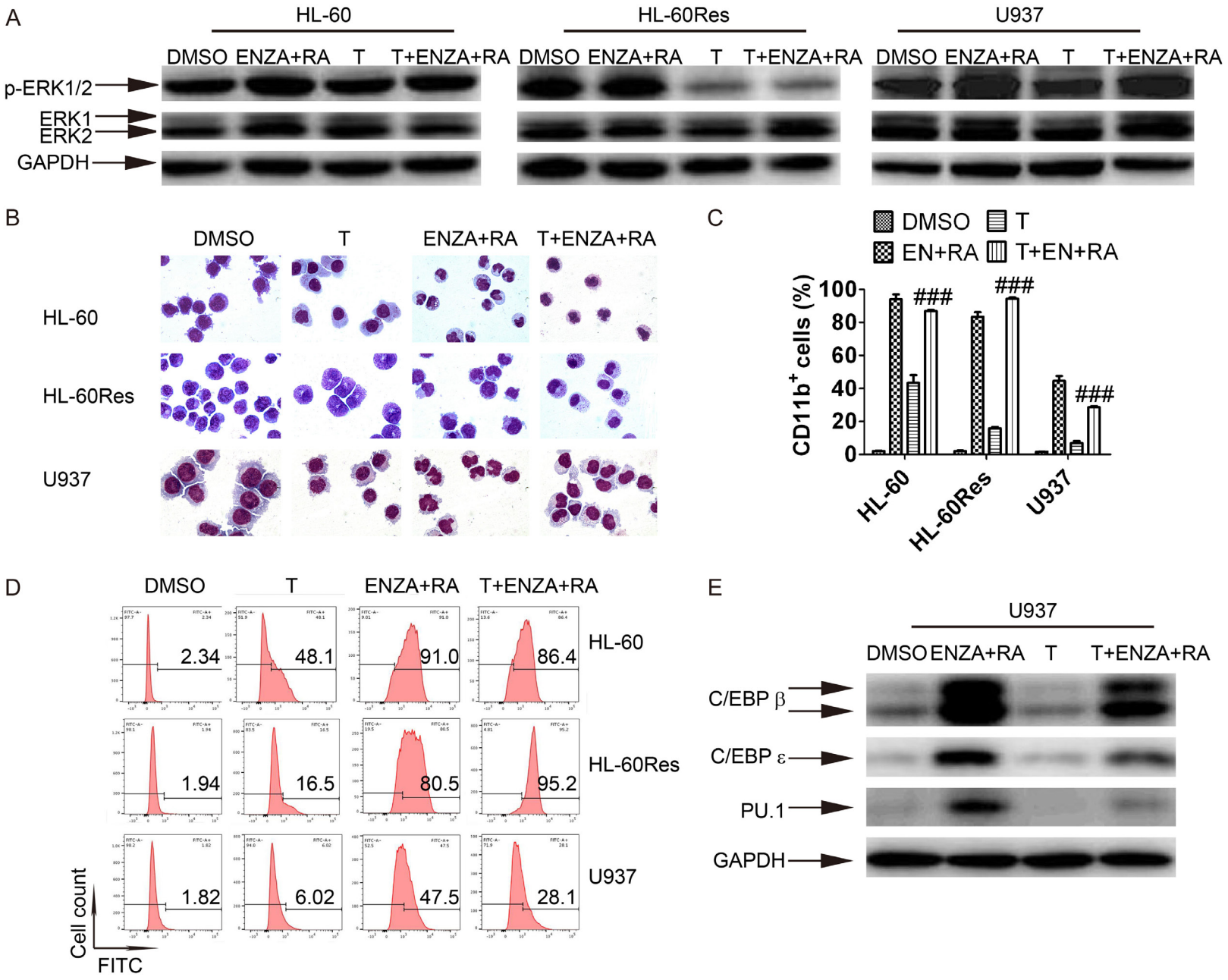


Figure 5. Enz-ATRA or PKCβ-inhibitor/ATRA treatment increases the protein levels of C/EBPβ, C/EBPε and PU.1, while enz-ATRA treatment activates MEK/ERK pathway only in U937 cells. HL-60 cells (upper left) were treated with 2 μM of enzastaurin (EN) and/or 0.1 μM of ATRA (RA) for 24 h. For the PKCβ inhibitor, 500 nM was used in HL-60 cells for 24 h (lower right). U937 cells (lower left) were treated with 2 μM of enzastaurin (EN) and/or 1 μM of ATRA (RA) for 6, 12 or 24 h. HL-60Res (upper right) cells were treated with 2 μM of enzastaurin (EN) and/or 1 μM of ATRA (RA) for 48 or 72 h. The same membrane incubated with the antibodies to phosphorylated Erk1/2 or MEK1/2 was stripped and followed by detection of MEK and ERK1/2. Since diverse time points for collecting protein were used, each has the expression of GAPDH as internal control. Similar results were obtained in three independent experiments.

induced differentiation in HL-60Res cells as evaluated by morphological analysis (**Figure 6B**) and the content of $CD11b^+$ cells (**Figure 6C**, **6D**). Therefore, MEK/ERK signal pathway may not regulate enz-ATRA treatment-triggered differentiation in HL-60 and HL-60Res cells.

Akt activation positively regulates enz-ATRA-induced differentiation in HL-60Res cells by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1

In addition to MEK/ERK, PI3K/AKT is another signaling pathway that has been demonstrated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HL-60
HL-60Res
U937
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EN+RA
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CD11b+ cells (%)
100
80
60
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###
HL-60
HL-60Res
U937
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2.34
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C/EBP ε
PU.1
GAPDH

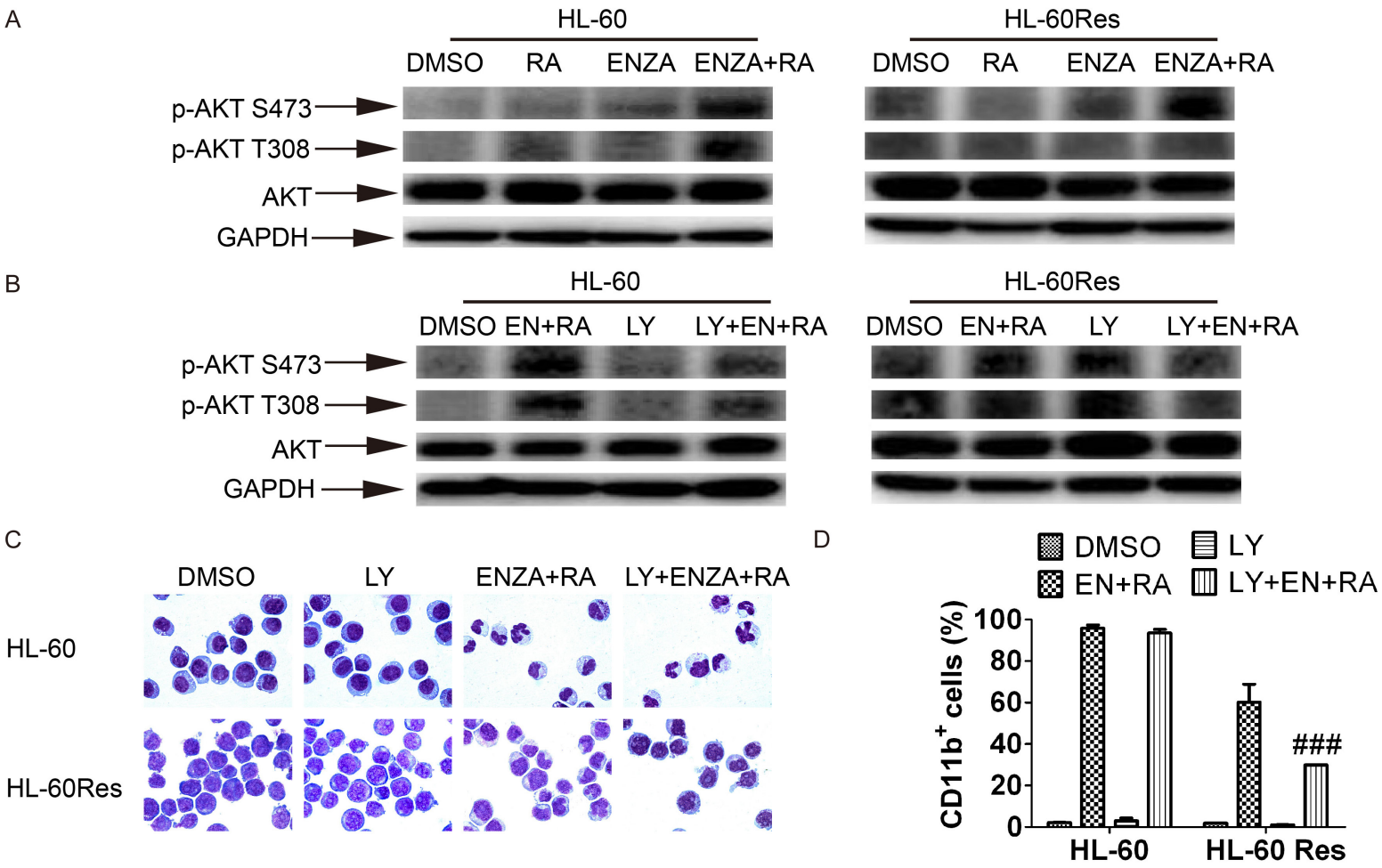
Figure 6. MEK/ERK inhibition suppresses enz-ATRA-triggered differentiation and restores the protein levels of C/EBPβ, C/EBPε and PU.1 only in U937 cells. HL-60, HL-60Res and U937 cells were pretreated with 0.01 μM, 0.1 μM and 5 μM of trametinib, respectively, for 2 h. A. The attenuation of MEK activation by trametinib (T) was detected by western blotting analysis of phosphorylated ERK1/2 in HL-60, HL-60Res and U937 cells with indicated treatments for 24, 48 or 4 h, respectively. The same membrane incubated with the antibodies to phosphorylated Erk1/2 was stripped and followed by detection of ERK1/2. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments. B. Effects of trametinib on morphologies in HL-60, HL-60Res and U937 cells incubated with the indicated drugs for 4, 10 or 3 d, respectively. The magnification is 1,000×. One representative experiment among three independent assays is shown. Similar results were obtained in three independent experiments. C. Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4, 6 or 3 d, respectively. Each value represents the mean ± SD of three independent measurements. ###*P*<0.001, as compared with 2EN+RA in U937 and HL-60Res cells or 2EN+0.1RA in HL-60 cells. D. Representative histograms of flow-cytometric analysis of CD11b expression in HL-60, HL-60Res and U937 cells with the indicated treatment for 4, 6 and 3 d, respectively. The percentages of $CD11b^+$ cells are shown in the corresponding panels. E. The protein levels of C/EBPβ, C/EBPε and PU.1 in U937 cells with the indicated drugs for 24 h, were assayed by western blotting. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments.

to be essential for ATRA-induced differentiation in HL-60 cells [29]. Phosphorylation of Ser473 or Thr308 is essential for activation of Akt [30]. In HL-60 cells, ATRA treatment for 48 h phosphorylated Akt at Ser473 and Thr308 while enz-ATRA treatment enhanced ATRA-promoted phosphorylation of both sites. In HL-60Res cells, ATRA did not enhance the phosphorylation of Akt, but enz-ATRA treatment for 72 h increased the phosphorylation of Akt at Ser473 (**Figure 7A**). LY294002, a PI3K inhibitor, did attenuate the activation of Akt in both cell lines (**Figure 7B**). However, LY294002 suppressed enz-ATRA-induced differentiation in HL-60Res cells but not in HL-60 cells as determined by morphological analysis (**Figure 7C**) and the content of $CD11b^+$ cells (**Figure 7D**, **7E**). Moreover, with LY294002 pretreatment, the protein levels of C/EBPβ, C/EBPε and PU.1 that were enhanced by enz-ATRA in HL-60Res cells were significantly reduced (**Figure 7F**). Therefore, AKT was not involved in enz-ATRA treatment-triggered differentiation in HL-60 cells. However, enz-ATRA treatment induced differentiation in HL-60Res cells via Akt modulation of the protein levels of C/EBPβ, C/EBPε and PU.1.

Discussion

In the present study, we demonstrated that enzastaurin enhanced ATRA-induced differentiation in HL-60, U937 and non-APL AML primary cells as well as reversed ATRA-resistance in HL-60Res cells. As mentioned above, these three cell lines are all without *NPM1* mutation, which is a marker of ATRA sensitivity [17, 18]. As ATRA-resistant cells, HL-60Res cells may better reflect the clinical effect of ATRA and enz-ATRA in non-APL AML patients. Moreover, the concentration of enzastaurin that we used in the present study is clinically achievable [19]. Taken together, our findings indicate that a combination of enz-ATRA may provide a potential therapeutic strategy for some AML patients. In our present study, for patient-derived AML primary cells, in four out of five AML-M4 and AML-M5 samples, enzastaurin enhanced ATRA-induced differentiation. Hence, a combination of enz-ATRA may be effective in AML-M4 and AML-M5 patients. However, we also observed that the effectiveness of enz-ATRA in AML primary cells might be associated with ATRA sensitivity. Therefore, the association of sensitivity to enz-ATRA treatment with AML subtype or ATRA sensitivity needs to be further surveyed in a large number of patient samples. Due to the limited number of patient samples, the association of sensitivity to enz-ATRA treatment with age, gender, chromosomal and genetic changes requires further investigation.

PKC is a family of serine/threonine kinases, consisting of 13 isozymes that play a crucial role in regulation of proliferation, differentiation, apoptosis, cell migration and gene expression. However, the role of different PKC isozymes in granulocytic differentiation is quite controversial. For ATRA-induced granulocytic differentiation in HL-60 cells, PKCα and PKCβII are activated and positively regulate granulocytic differentiation [31]. However, Zauli et al reported that only PKCα and PKCζ but not PKCβII or PKCβI showed significant modification upon ATRA treatment in HL-60 cells [32]. Kambhampati et al showed that only PKCδ was activated with ATRA treatment in HL-60 cells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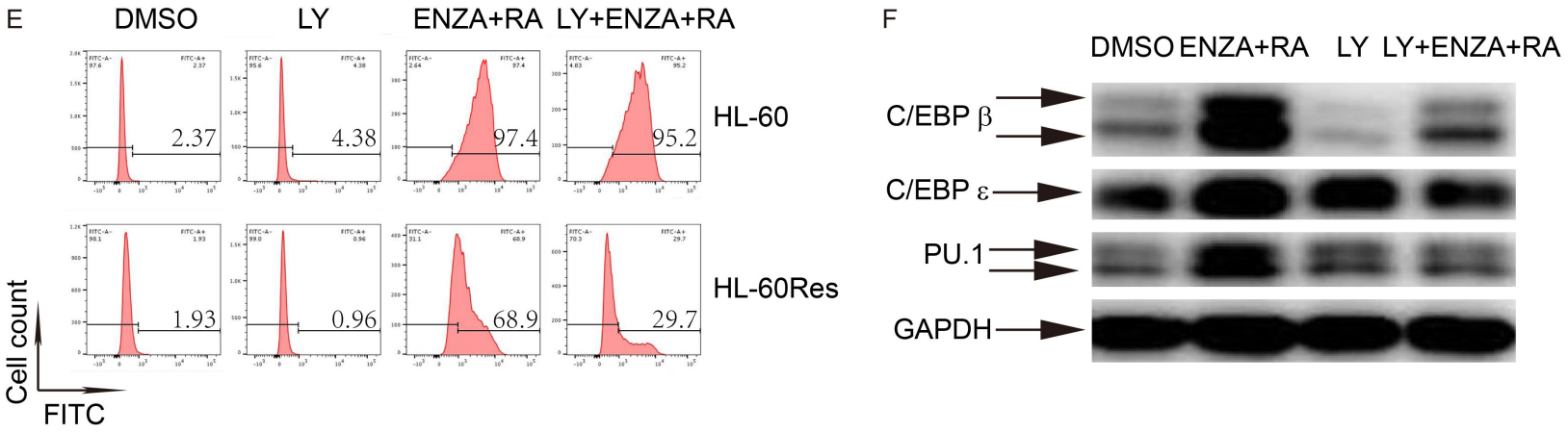

Figure 7. AKT activation is required for enz-ATRA-induced differentiation and enhanced protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60Res cells. A. HL-60 cells were treated with 2 μM of enzastaurin (EN) and/or 0.1 μM of ATRA (RA) for 48 h. HL-60Res cells were treated with 2 μM of enzastaurin (EN) and/or 1 μM of ATRA (RA) for 72 h. The activation of AKT was evaluated by western blotting analysis of phosphorylated AKT at serine 473 and threonine 308. The same membrane incubated with anti-phospho-AKT was stripped and followed by detection of AKT. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments. B. The attenuation of AKT activation by LY294002 (LY) was detected by western blotting analysis of phosphorylated AKT in HL-60 and HL-60Res cells with indicated treatments for 48 or 72 h, respectively. The same membrane incubated with the anti-phospho-AKT was stripped and followed by detection of AKT. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments. C. The effects of LY294002 on morphologies in HL-60 and HL-60Res cells incubated with the indicated drugs for 4 or 10 d, respectively. The magnification is 1,000×. One representative experiment among three independent assays is shown. Similar results were obtained in three independent experiments. D. Differentiation was also evaluated by flow-cytometric analysis of CD11b expression in HL-60 and HL-60Res cells with the indicated treatment for 4 or 6 d, respectively. Each value represents the mean ± SD of three independent measurements. ###$P<0.001$, as compared with 2EN+RA in HL-60Res cells. E. Representative histograms of flow-cytometric analysis of CD11b expression in HL-60 and HL-60Res cells with the indicated treatment for 4 or 6 d, respectively. The percentages of $CD11b^+$ cells are shown in the corresponding panels. F. The protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60Res cells with the indicated drugs for 72 h, were assayed by western blotting. The expression of GAPDH was evaluated as internal control. Similar results were obtained in three independent experiments.

[33]. For myeloid differentiation, it has been suggested that PKCα is not required for granulocytic differentiation because its mRNA and protein are decreased with ATRA treatment in HL-60 cells and are undetectable in peripheral blood neutrophils [34]. However, PLCγ2 and PKC are demonstrated to be crucial upstream signals that modulate myelopoiesis via G-CSF [35]. In the present study, inhibition of PKCβ by either enzastaurin or another PKCβ inhibitor enhanced ATRA-induced differentiation in HL-60 cells. This finding indicates that PKCβ may negatively regulate ATRA-induced differentiation in HL-60 cells. Further study showed that enzastaurin or a PKCβ inhibitor each increased ATRA-enhanced protein levels of C/EBPβ, C/EBPε and PU.1 in HL-60 cells. This suggests that PKCβ inhibition may control enzastaurin-enhanced differentiation in HL-60 cells by modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. PKC positively or negatively regulates the transcriptional activity of C/EBPβ via phosphorylation at different sites [36, 37]. In addition, C/EBPβ can trigger expression of C/EBPε and PU.1 during ATRA-triggered differentiation in APL cells [22, 23]. PU.1 can also directly activate the transcription of C/EBPε [38]. Therefore, a PKCβ inhibition-C/EBPβ-PU.1-C/EBPε cascade may occur during enz-ATRA-induced differentiation in HL-60 cells. In our present study, PKCβ was inhibited by enz-ATRA in HL-60Res cells, whereas it was not suppressed in U937 cells. Moreover, another PKCβ inhibitor did not mimic the effect of enzastaurin to enhance ATRA-induced differentiation in either cell lines. Thus, PKCβ may not be involved in enz-ATRA-induced differentiation in these two cell lines. In fact, PKCβ-independent effects of enzastaurin are not rare, as they have been reported in many previous studies [16, 39-41].

Compared with that of ATRA, the phosphorylation levels of MEK and ERK were enhanced with enz-ATRA treatment only in U937 cells. Moreover, a specific inhibitor of MEK suppressed both enz-ATRA-induced differentiation and the protein levels of C/EBPβ, C/EBPε and PU.1 only in U937 cells. Therefore, MEK/ERK modulation of the protein levels of C/EBPβ, C/EBPε and PU.1 was involved in enz-ATRA-induced differentiation only in U937 cells, but not HL-60 cells or HL-60Res cells. Consistent with our present study, staurosporine, the parent compound of enzastaurin, has been previously shown to enhance ATRA-induced differentiation in U937 cells, also via MEK/ERK-mediated modulation of C/EBPs [42]. MEK and ERK have been demonstrated to promote the expression of C/EBPβ and regulate the activity of C/EBPβ and PU.1 [43-45]. Since there is interaction among C/EBPβ, C/EBPε and PU.1 [22, 23, 38], a MEK-ERK-C/EBPβ-PU.1-C/EBPε cascade may occur during enz-ATRA-induced differentiation in U937 cells.

Akt can positively or negatively control differentiation of leukemia cells depending on cell type and differentiation inducer [31, 46]. In HL-60 cells, Akt activation is required for ATRA-induced differentiation [29]. In the present study, although Akt was activated by enz-ATRA treatment, inhibition of Akt did not suppress enz-ATRA-triggered differentiation in HL-60 cells. Thus, the involvement of Akt in enz-ATRA-triggered differentiation in HL-60 cells was excluded. In HL-60Res cells, Akt was activated by enz-ATRA treatment, and an Akt inhibitor suppressed both differentiation and the protein levels of C/EBPβ, C/EBPε and PU.1. Therefore, our findings demonstrate that Akt activation was involved in enz-ATRA-induced differentiation in HL-60Res cells via modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Akt induces transcriptional activity and expression of C/EBPβ and PU.1 [47-50]. In addition, C/EBPβ can trigger the expression of C/EBPε and PU.1 [22, 23]. Collectively, these findings suggest that an Akt-C/EBPβ-PU.1-C/EBPε cascade may occur during enz-ATRA-induced differentiation in HL-60Res cells.

Taken together, our present study revealed that a clinically-achievable concentration of enzastaurin enhanced ATRA-induced differentiation in HL-60, U937, and non-APL AML primary cells, as well as reversed ATRA-resistance in HL-60Res cells. Furthermore, we elucidated that PKCβ inhibition, MEK/ERK activation, and Akt activation were involved in enz-ATRA-induced differentiation in HL-60, U937 and HL-60Res cells, respectively, via modulation of the protein levels of C/EBPβ, C/EBPε and PU.1. Collectively, our present findings may contribute to the development of novel therapeutic strategies for some AML patients.

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Disclosure of conflict of interest

None.

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