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ORIGINAL ARTICLE

Salt-inducible kinase inhibition sensitizes human acute myeloid leukemia cells to all-trans retinoic acid-induced differentiation

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Abstract

Differentiation therapies with all-trans retinoic acid (ATRA) have been successful in treating acute promyelocytic leukemia, a rare subtype of acute myeloid leukemia (AML). However, their efficacy is limited in the case of other AML subtypes. Here, we show that the combination of ATRA with salt-inducible kinase (SIK) inhibition significantly enhances ATRA-mediated AML differentiation. SIK inhibition augmented the ability of ATRA to induce growth inhibition and G1 cell cycle arrest of AML cells. Moreover, combining ATRA and SIK inhibition synergistically activated the Akt signaling pathway but not the MAPK pathway. Pharmacological blockade of Akt activity suppressed the combination-induced differentiation, indicating an essential role for Akt in the action of the combination treatment. Taken together, our study reveals a novel role for SIK in the regulation of ATRA-mediated AML differentiation, implicating the combination of ATRA and SIK inhibition as a promising approach for future differentiation therapy.

Keywords Salt-inducible kinase · ATRA · Differentiation · Acute myeloid leukemia · Akt

Introduction

Acute myeloid leukemia (AML), the most common adult leukemia, is characterized by defective differentiation, aberrant proliferation, and inappropriate survival of clonal precursor myeloid cells [1]. Although remission can be achieved in most de novo AML patients, the leukemia will recur with poor outcomes. The only exception is treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) differentiation therapy. In APL, treatment with ATRA alone or in combination with anthracycline-based chemotherapy, results in myeloid differentiation of the leukemic blasts and leads to a high rate of complete remission and the long-term survival [2]. However, ATRA is not clinically useful for the non-APL AML subtypes. Therefore, there is an urgent need to develop new differentiation therapies to improve the efficiency of ATRA against a wide range of other myeloid malignancies.

Salt-inducible kinase (SIK) is a subfamily of the AMP-activated protein kinase (AMPK) family [3, 4]. The SIK family kinases contain three isoforms (SIK1, SIK2, and SIK3) that have been recognized for their role in the regulation of cellular metabolism, growth, and apoptosis. Growing evidence has demonstrated that SIKs play an important role in the regulation of a variety of cellular processes required for tumor development, such as cell-cycle progression, mitosis, and lipogenesis [5, 6]. Overexpression of SIK2 and SIK3 has been identified to facilitate cell cycle progression and cell proliferation in ovarian cancer [7, 8]. SIK2 may also promote omental ovarian cancer metastasis by activating the PI3K/Akt pathway [9].

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Recently, a couple of studies have discovered functional roles for SIKs in AML. In mixed -lineage leukemia (MLL) fusion AML subtypes that harbor high myocyte enhancer factor 2C (MEF2C) activity, SIK inhibition has antiproliferative effects [10–12]. Specifically, SIK3 inhibition has been shown to preferably inhibit MLL-fusion AML cell growth and suppress AML progression in vivo [10, 12]. These findings suggest that targeting SIKs is a novel therapeutic strategy for AML.

In the present study, we describe a novel role for SIKs in regulating ATRA-mediated differentiation of myeloid leukemia cells. We observed that SIK inhibition effectively exacerbate ATRA-induced differentiation in AML cells. This enhanced differentiation might be associated with the activation of Akt signaling rather than MAPK pathway. Pharmacological blockade of Akt activity significantly decreased the combined differentiation effect of ATRA and SIK inhibition. These findings reveal an unrecognized role of SIK in the regulation of AML differentiation and identify specific molecular targets for differentiation-inducing approaches to a large proportion of myeloid leukemias.

Materials and methods

Reagents

SIK inhibitors, HG-9-91-01 and YKL-05-099, and the AKT inhibitor MK2206 were obtained from Selleckchem Chemicals (Houston, TX). Antibodies against SIK1, SIK2, p21, CDK4, CDK6, Cyclin D1, Rb, phospho-Rb (Ser807/811), ERK, Phospho-ERK, JNK, Phospho-JNK, P38, Phospho-P38, phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-GSK3β (Ser9), GSK3β, phospho-mTOR (Ser2448), mTOR, phospho-p70S6K (Thr389), and phospho-p70S6K (Ser271) were obtained from Cell Signaling Technology (Danvers, MA). Phycoerythrin-labeled antibody against CD11b was purchased from BD Biosciences (San Jose, CA). SIK3, Raf, and Phospho-Raf (Ser259) antibodies were purchased from Abcam (Cambridge, UK). GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise stated, other chemicals and reagents were obtained from Sigma (St. Louis, MO).

Cell culture

The human myeloid leukemia cell lines HL-60, NB4, U937, and THP-1 were all cultured in RPMI medium containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) at 37 °C in the presence of 5% CO_2.

Differentiation

Cell morphological features, nitroblue tetrazolium chloride (NBT) reduction and cell-surface CD11b expression assay were measured as we reported before [13, 14].

Analyses of cell growth, apoptosis, and cell cycle distribution

For cell growth assay, viable cells were counted using trypan blue exclusion method as previously described [13]. For cell cycle analysis, cells were collected and fixed with cold 70% ethanol overnight at 4 °C. The fixed cells were then washed with PBS, treated with 1% RNase A for 15 min at 37 °C, and stained with 50 μg/ml propidium iodide (PI). Cells were washed and analyzed by flow cytometry. Annexin V/PI (BD Pharmingen) double-staining analysis of apoptosis was conducted according to the manufacturer's instructions.

Lentiviral infections

For knockdown of SIK proteins, the lentiviral-based small hairpin RNAs (shRNAs) targeting respective SIK isoforms (SIK1 target sequence:5′-TGACAGTTGTCTGACCTTCT-3′; SIK2 target sequence:5′-GCAGTTGTTGTATGAACAA-3′; SIK3 target sequence: 5′-CCACAGAATGTGAGCATTT-3′) were obtained from Genechem (Shanghai, China). AML cell lines were spin infected with the viral supernatant concentrated in PEG and 4 μg/mL polybrene. Stable cell lines were generated by selection with puromycin (4 μg/mL).

Real-time quantitative PCR assay

Quantitative real-time PCR (qRT-PCR) analysis was performed as previously described [13]. The primers used for PCR are listed in Table 1.

Table 1 Primers list

Genes	Primer 1 (Forward, 5′→3′)	Primer 2 (Reverse, 5′→3′)
SIK1	CTCCGGGTGGGTTTTTACGAC	CTGCG TTTTGGTGACTCGATG
SIK2	GCCTTTTCATTTCCAGCATC	GTCTCCAGCCCTTCGTCA
SIK3	TGGGGAAAATGAGGAATGTG	CGTAGATGGATAGCAAGAGGAG
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Western blot analysis

Western blot analyses were performed on cells after desired treatments according to previously described procedures [13].

Statistical analysis

Data presented here represent at least three independent experiments. All data are presented as mean ± standard deviation (SD). Statistical comparisons were done by the Student's *t* test or one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

Results

SIK inhibition sensitizes AML cells to ATRA-mediated myeloid differentiation

To assess the role of SIK inhibition in the differentiation of AML cells, two selective SIK inhibitors HG-9-91-01 and YKL-05-099 were used at nontoxic concentrations to examine their differentiation-inducing effects on HL-60 cells via detection of CD11b differentiation marker. It was showed that HG-9-91-01, when used at a higher dose (1 μM), led to a moderate increase in the expression of CD11b (Fig. 1a, b). Intriguingly, both HG-9-91-01 and YKL-05-099 significantly sensitized HL-60 cells to differentiate in the presence of 10 nM of ATRA, which alone have only weak differentiation-inducing effects (Fig. 1a–d). CD11b expression in HL-60 cells was 75% with the combination of ATRA and HG-9-91-01 (0.5 μM), but only 11% and 9% with ATRA and HG-9-91-01, respectively. Similarly, the percentage of CD11b-positive cells increased from 8% in the 1 μM YKL-05-099-treated group to 71% in the ATRA and YKL-05-099 combination group. To confirm the differentiating effect of SIK inhibitors and ATRA, we used the NBT reduction assay to quantify the number of functionally mature myeloid cells. Consistent with CD11b expression, we observed a significant enhancement in NBT-positive HL-60 cells after combination treatment as compared to single-agent treatment (Fig. 1e and Supplementary Fig. S1). The synergistic interactions between ATRA and SIK inhibitors to induce differentiation can also be seen by morphological assessment as the combination of these agents caused morphological evidence of myeloid differentiation with decreased nucleus–cytoplasm ratio and condensed, distorted and stab form nuclei (Fig. 1f).

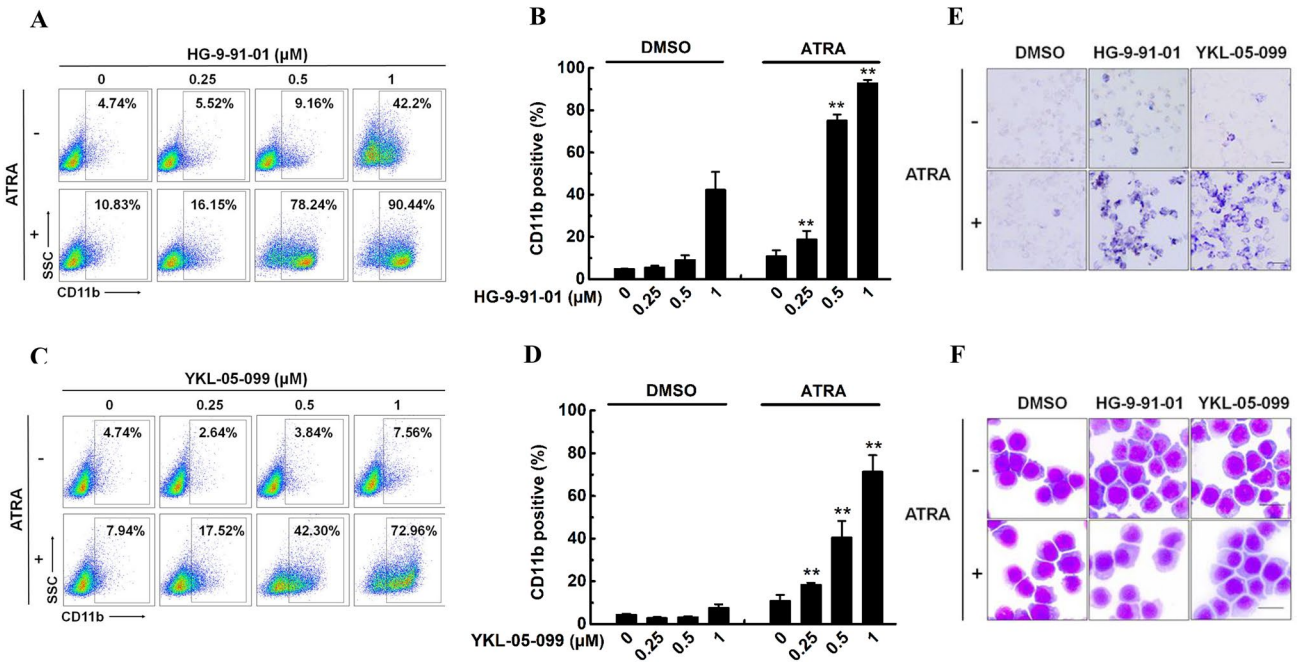

Fig. 1 Effect of SIK inhibitors on ATRA-mediated differentiation in HL-60 cells. **a**–**d** Cells were treated with the indicated concentrations of HG-9-91-01 or YKL-05-099, in the presence or absence of 10 nM ATRA for 72 h. The percentage of CD11b-positive cells was measured by flow cytometry. **a**, **c** Representative flow cytometric dot plots of CD11b-labeled cells and **b**, **d** graphical representation of three replicates. **e**, **f** HL-60 cells were treated with 10 nM ATRA alone or in combination with 0.5 μM HG-9-91-01 or 1 μM YKL-05-099 for 72 h. NBT reduction (**e**) and Giemsa's staining morphology (**f**) were measured. Scale bars, 20 μm. Data represent mean ± SD of three independent experiments. $^{**}p < 0.01$ versus single treatments (ATRA or SIK inhibitors)

In addition to HL-60 cells, we also observed that HG-9-91-01 had the ability to enhance ATRA-mediated differentiation in other AML cell lines of promyelocytic (NB4) and monoblastic (U937 and THP-1) origin. The combination of ATRA and HG-9-91-01 synergistically induced differentiation in these cells, as evidenced by maturation morphologic features (Fig. 2a) and increased NBT reduction (Fig. 2b and Supplementary Fig. S2) and percentage of CD11b-positive cells (Fig. 2c, d), with the latter being free of CD14 (Supplementary Fig. S3), a monocytic differentiation marker. Together, these results suggest that SIK inhibition significantly potentiated the differentiating effects of ATRA on AML cells.

SIK inhibition enhances ATRA-mediated growth inhibition and cell cycle arrest

We next sought to determine the potential antiproliferative effects of ATRA combined with SIK inhibition. Results showed that the combination of ATRA plus HG-9-91-01 synergistically induced growth arrest of HL-60 and THP-1 cells in a concentration- and time-dependent manner (Fig. 3a, b). Minimal induction of apoptosis was observed in this case (Fig. 3c, d). Conversely, the combination regimen led to effects on the cell-cycle progression of AML cells. We observed that the combination of ATRA and HG-9-91-01 was more efficient at inducing G1 cell cycle arrest of HL-60 and THP-1 cells than any of these agents used alone (Fig. 3e). Further investigations showed that ATRA combined with HG-9-91-01 modulated levels of cell cycle-related molecules, evidenced by upregulation of p21 protein expression and downregulation of G1 phase-related

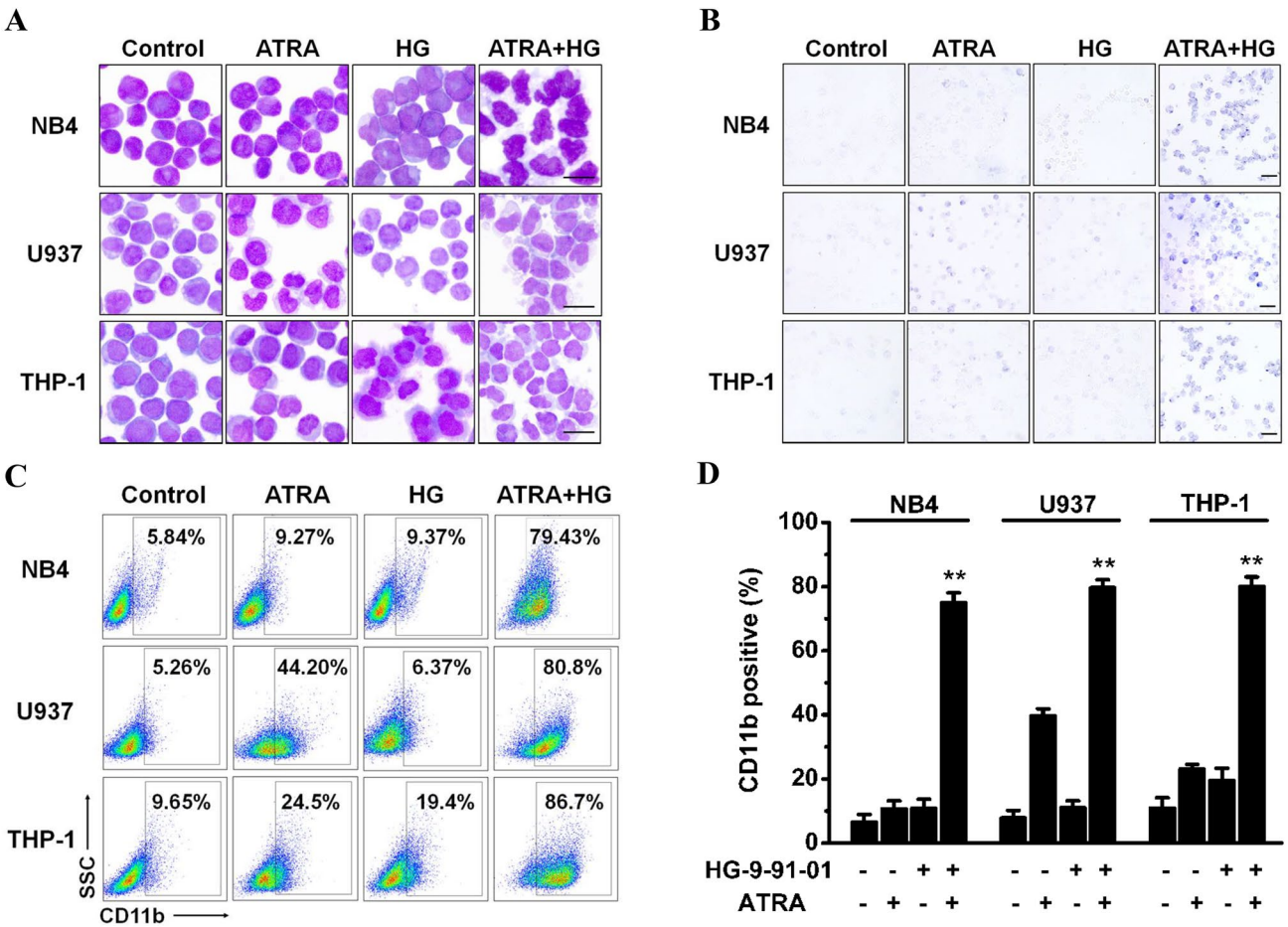

Fig. 2 Effect of SIK inhibitors on ATRA-induced differentiation of other AML cell lines. Cells were treated with HG-9-91-01 (0.25 μM for U937, 0.5 μM for THP-1, and 1 μM for NB4), ATRA (10 nM for U937 and THP-1 and 1 nM for NB4), or a combination for 72 h. **a**, **b** Giemsa's staining morphology (**a**) and NBT reduction (**b**) were measured. Scale bars, 20 μm. **c**, **d** Flow cytometric analysis of CD11b-labeled cells. **c** Representative flow cytometric dot plots of CD11b-labeled cells and **d** graphical representation of three replicates. Data represent mean ± SD of three independent experiments. $^{**}p < 0.01$ versus single treatments (ATRA or HG-9-91-01)

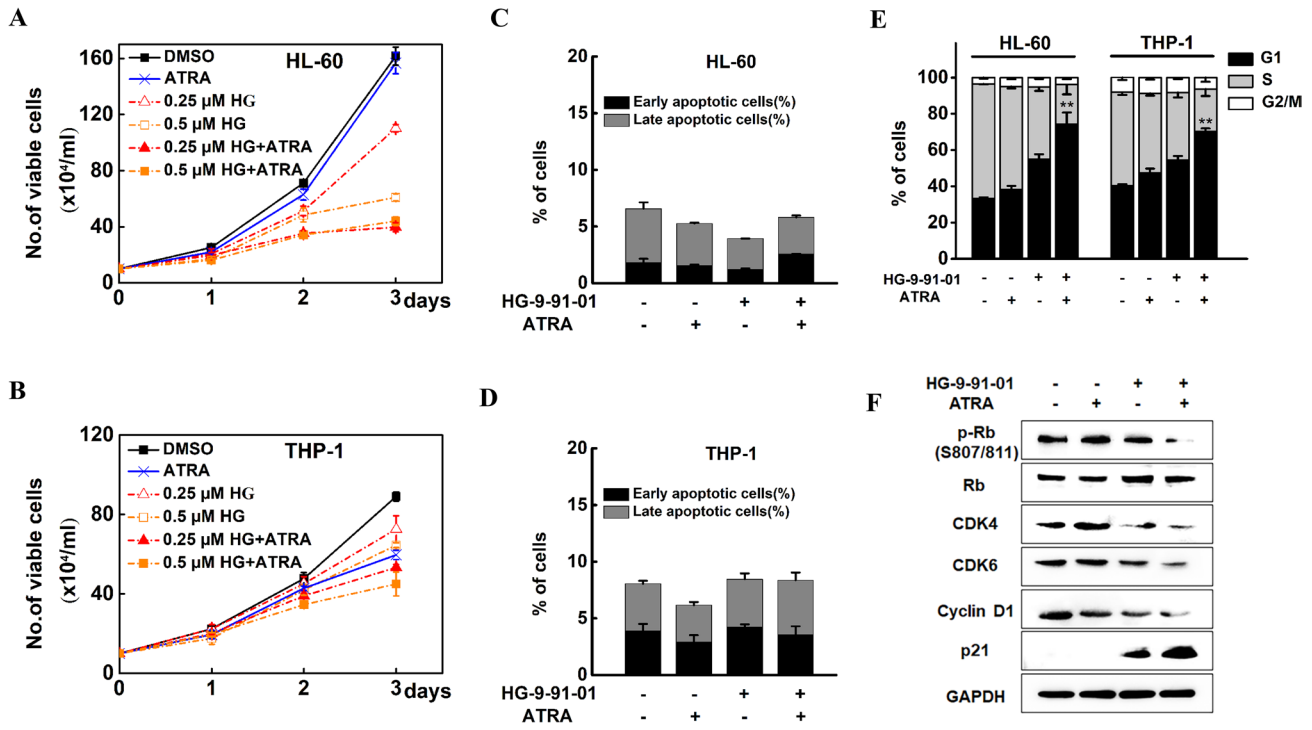

Fig. 3 Effect of SIK inhibitors and ATRA on AML cell growth and cell cycle progression. **a**, **b** Cells were treated with ATRA (10 nM) and HG-9-91-01 (HG) or vehicle and growth suppression was measured by the trypan blue dye exclusion method. **c**, **d** Cells were treated with ATRA (10 nM) and HG-9-91-01 (0.5 μM) for 24 h. Apoptosis was detected by flow cytometric annexin V/propidium iodide (PI) binding analysis. Early apoptotic cells are annexin V-positive and PI-negative (Annexin V-FITC$^+$/PI$^-$), and late apoptotic cells are annexin V/PI-double-positive (Annexin V-FITC$^+$/PI$^+$). **e**, **f** Cells were incubated with 10 nM ATRA alone or in combination with 0.5 μM HG-9-91-01 for 24 h. DNA content was assayed by flow cytometry (**e**), and HL-60 cell lysate was analyzed by western blot analysis (**f**). $^{**}p < 0.01$ versus single treatments (ATRA or HG-9-91-01)

checkpoint proteins cyclin D1, CDK4, CDK6 and phosphorylated retinoblastoma (Rb) protein expression (Fig. 3f). Consistent with the cell cycle block, the expression of cell cycle-related molecules is regulated more by the combined treatment of ATRA and HG-9-91-01 as opposed to single-agent treatment.

These results indicate that ATRA synergizes with SIK inhibition in inducing AML cell differentiation, a process that is coupled to the growth inhibition and to the induction of cell cycle arrest.

Reduction of SIK enhances ATRA-induced differentiation of AML cells

Though we utilized well-established SIK inhibitors, no pharmacological inhibitors are devoid of off-target effects. Therefore, genetic experiments were conducted to validate the role of SIK in AML differentiation. We silenced all three SIK isoforms individually in three AML cell lines (HL-60, THP-1, and NB4) with relatively high endogenous expression of the SIK isoforms (Supplementary Fig. S4). shRNA-mediated SIK1 knockdown led to the downregulation of mRNA but not protein levels (Supplementary Fig. S4); this condition was, thus, excluded in further analysis. As shown in Fig. 4a, b, SIK2 and SIK3 were significantly knocked down by their respective shRNAs at both the mRNA and protein levels in HL-60, THP-1, and NB4 cells. We found that knockdown of either SIK2 or SIK3 resulted in slight growth inhibition without increase in the basal level of differentiation (Fig. 4c–e, Supplementary Fig. S5). However, these SIK-deficient AML cells are sensitized to ATRA-induced differentiation supporting our pharmacological data that SIK plays an important role in ATRA-mediated differentiation.

MAPK pathway might be dispensable for myeloid differentiation induced by the combination of ATRA and SIK inhibition

To elucidate the mechanisms underlying the synergy between ATRA and SIK inhibition, we determined whether MAPK pathway was involved in the combination treatment-induced differentiation, given that ATRA-induced differentiation is driven by a sustained MAPK signal [15, 16]. We first evaluated the activation of MAPK signaling by determining phosphorylated protein levels of Raf, MEK, ERK, P38, and

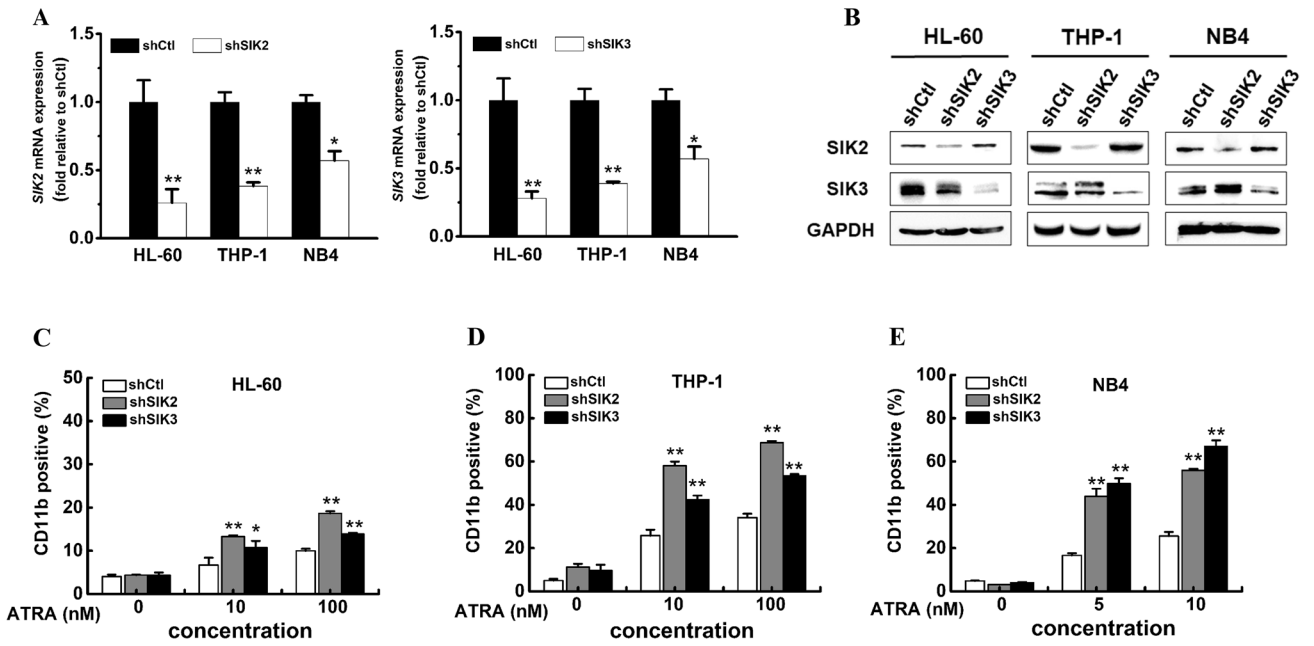

Fig. 4 Genetic evidence of the role of SIK in AML differentiation. **a**, **b** HL-60, THP-1, and NB4 cells were stably transduced with recombinant lentiviruses expressing either empty vector (shCtl), SIK2, or SIK3 shRNAs. The mRNA levels and protein levels of SIK2 and SIK3 were detected by real-time PCR (**a**) and western blot (**b**), respectively. **c**–**e** Transduced HL-60, THP-1, and NB4 cells were incubated with the indicated concentrations of ATRA for 72 h. The percentage of CD11b-positive cells was analyzed by flow cytometry. $^{*}p<0.05$, $^{**}p<0.01$ versus cells transduced with empty vector

JNK in HL-60 cells after ATRA and/or HG-9-91-01 treatment. Unexpectedly, ATRA, HG-9-91-01, or the combination of the two showed minimal effects on the tested MAPK proteins phosphorylation or expression (Fig. 5a). Furthermore, pretreatment with specific inhibitors of ERK, P38, and JNK did not affect the combination-induced cell differentiation (Fig. 5b). Thus, these results suggested that MAPK pathway might not be essential for myeloid differentiation induced by the combination of ATRA and SIK inhibition.

Combining ATRA and SIK inhibition leads to an enhanced activation of Akt signaling pathway

Previous studies have demonstrated that SIKs played an important role in the regulation of PI3K/Akt pathway [6, 9]. In addition, as activation of Akt signaling may contribute to ATRA-mediated differentiation [17, 18], we next determined the effects of combined ATRA with SIK inhibition on the Akt signaling pathway in AML cells. Although ATRA did not have a substantial effect on Akt phosphorylation, HG-9-91-01 resulted in an increase in the levels of Thr 308 and Ser 473 p-Akt (Fig. 6a), which was further enhanced by co-treatment with ATRA. We also observed that both ATRA and SIK inhibition induced an apparent increase in the levels of Ser 2448 p-mTOR, and Thr 389 and Ser 371 p70S6K; whereas, this increase was not reinforced by the combination treatment. However, serine 9 phosphorylation within GSK-3β, a well-characterized Akt substrate, was elevated more by the combined treatment of ATRA and SIK inhibition as opposed to single-agent treatment. Together, these results indicate that the Akt signaling pathway was significantly activated by the combination treatment.

To determine the functional role of Akt signaling in the combination-induced AML cell differentiation, we treated HL-60 cells with the Akt inhibitor MK2206 [19]. We found that pretreatment with MK2206 dramatically suppressed the combination-induced phosphorylation of Akt and its downstream effectors p70S6K and GSK-3β (Fig. 6b). As expected, MK2206 significantly inhibited the differentiating effects of the ATRA plus SIK inhibitor combination as measured using CD11b induction (Fig. 6c). Consistently, similar inhibitory effects of MK2206 on enhanced ATRA-mediated differentiation were further observed in SIK-deficient NB4 cells (Fig. 6d). Overall, these results support that the activation of Akt signaling contributes to myeloid differentiation induced by the combination of ATRA and SIK inhibition.

Discussion

ATRA-based differentiation therapy has been successful in the treatment of APL. However, most AML patients with other non-APL subtypes display resistance to ATRA-based

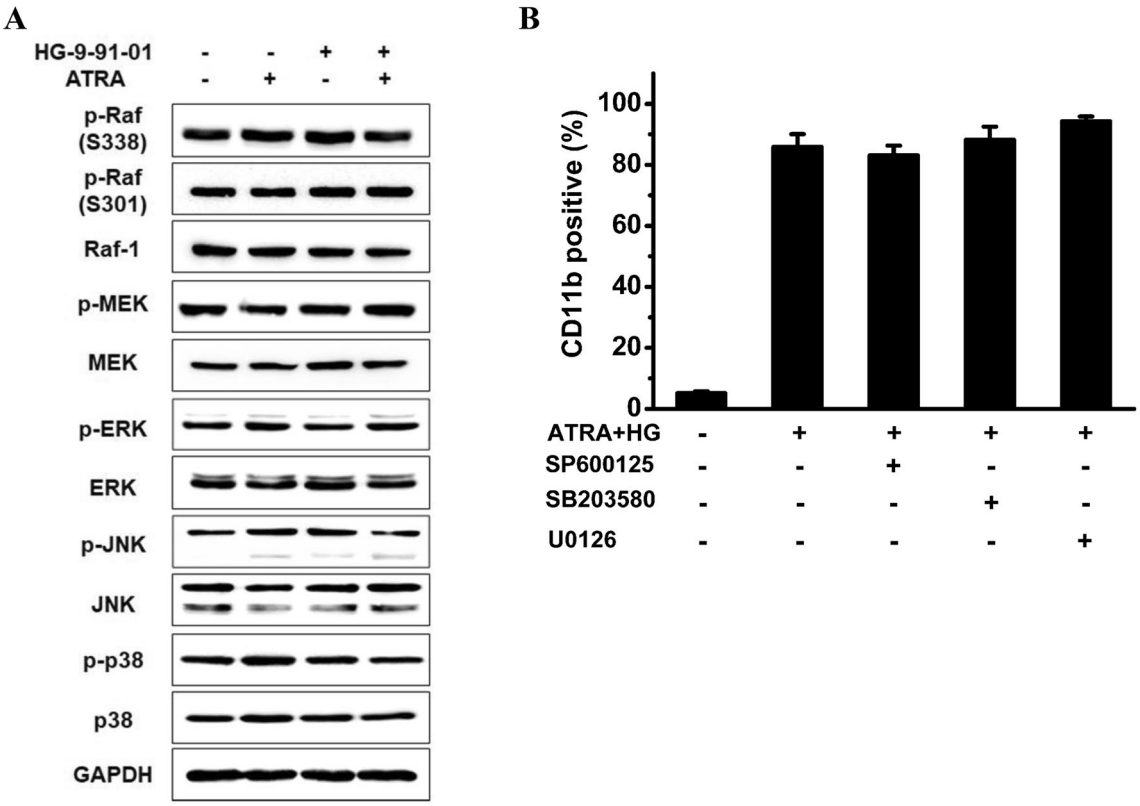

Fig. 5 Effect of ATRA and SIK inhibition on MAPK pathway. **a** HL-60 cells were treated with ATRA (10 nM), HG-9-91-01 (0.5 μM), or a combination for 24 h, and total cell lysates were assayed by western blotting. **b** After pretreatment with and without 10 μM U0126, 10 μM SB203580, or 10 μM SP600125 for 1 h, HL-60 cells were treated with 0.5 μM HG-9-91-01 combined with 10 nM ATRA for 72 h. CD11b expression was measured by flow cytometry

treatment. Here, to the best of our knowledge, we demonstrate for the first time that SIK is a negative regulator of ATRA-mediated AML differentiation. Inhibition of SIK through pharmacologic or genetic approaches significantly enhanced AML differentiation in a variety of AML cell lines. Of note, we found that the biological function of the combination of ATRA and SIK inhibition in inducing cell differentiation is driven by the activation of Akt signaling rather than MAPK pathway.

In this study, the combination of ATRA and SIK inhibitor was found to enhance growth inhibition and dramatically induce G1 cell cycle arrest in AML HL-60 and THP-1 cells. Induction of cell cycle arrest coincided with differentiation induced by ATRA combined with SIK inhibition, suggesting that the cell cycle arrest might be associated with the terminal differentiation of AML cells. Terminal differentiation is usually coupled with permanent exit from the cell cycle, and this process may require the coordinated activities of the Rb family of proteins and CDK inhibitors [20]. In addition, the ability of retinoids to induce cell-cycle arrest, through transcriptional regulation of various cell-cycle regulators, is critical for their therapeutic effects [21]. As expected, our further observations showed that the cell cycle arrest induced by the combination may be associated with an increase in p21 levels and reduction of cyclin D1, CDK4, CDK6, and phosphorylated Rb.

Numerous signaling pathways including MEK/ERK/MAP kinase and PI3K/Akt/mTOR cascades have been shown to play important roles in ATRA-induced AML differentiation [15–18]. However, our data indicated that MAPK activation might not be necessary for the biological effects of ATRA and SIK inhibition, as various MAPK proteins did not show changes in the phosphorylation status or expression levels during combination-induced cell differentiation. In turn, we observed that SIK inhibition alone increased the phosphorylation of Akt, which was further enhanced by co-treatment with ATRA. More importantly, pharmacological inhibition of Akt dramatically reduced cell differentiation by ATRA plus SIK inhibition, suggesting the important role of Akt activation in the differentiation caused by the combination. We further showed that combined ATRA with SIK inhibition treatment led to the activation of Akt's downstream targets, mTOR, and p70S6K, which has been reported to be activated by ATRA in APL cells [18].

However, modulation of mTOR and p70S6K activation did not parallel differentiation induced by the combination,

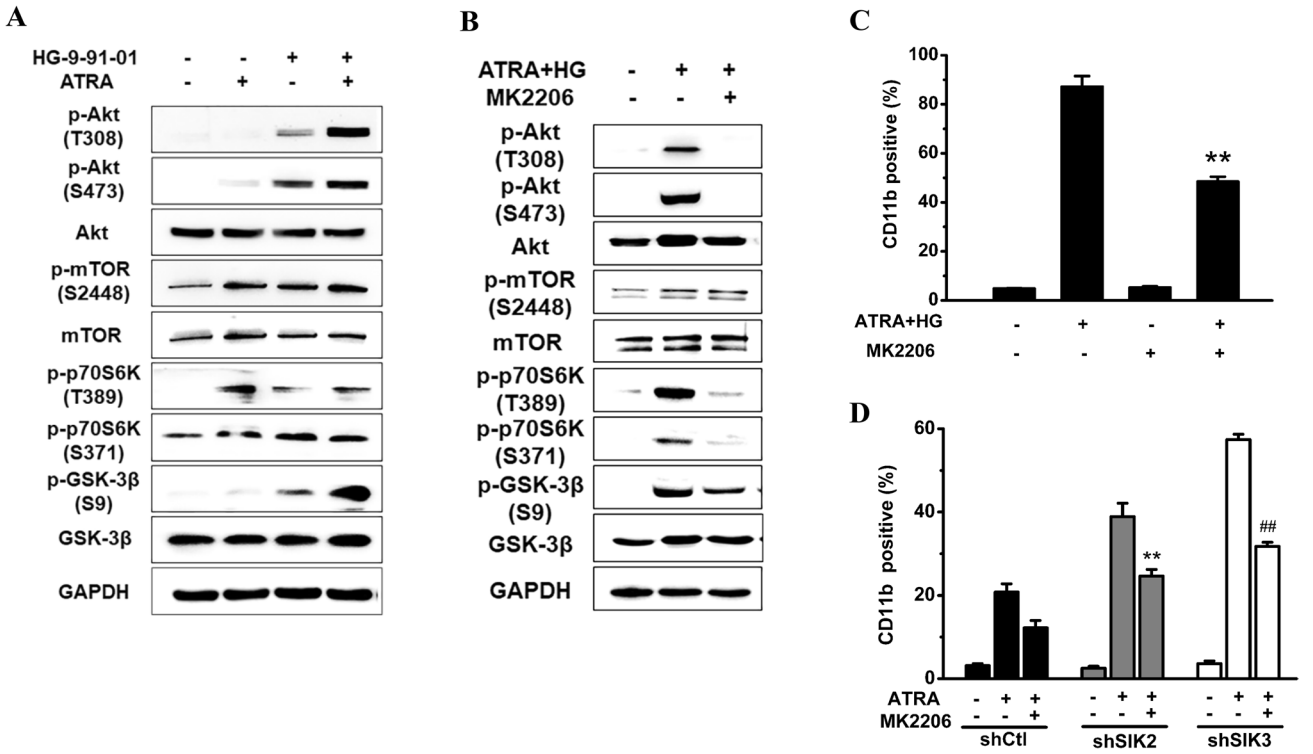

Fig. 6 SIK inhibition and ATRA activated the Akt signaling. **a** HL-60 cells were treated with ATRA (10 nM), HG-9-91-01 (0.5 μM), or a combination for 24 h, and total cell lysates were analyzed by western blot. **b** After pretreatment with or without 5 μM MK2206 for 1 h, HL-60 cells were treated with 0.5 μM HG-9-91-01 combined with 10 nM ATRA for 24 h, and total cell lysates were analyzed by western blot. **c** After pretreatment with or without 5 μM MK2206 for 1 h, HL-60 cells were treated with 0.5 μM HG-9-91-01 combined with 10 nM ATRA for 72 h, and CD11b expression was assayed by flow cytometry. $^{**}p < 0.01$ versus ATRA plus HG-9-91-01 treatment. **d** After pretreatment with or without 5 μM MK2206 for 1 h, transduced NB4 cells were treated with 10 nM ATRA for 72 h, and CD11b expression was assayed by flow cytometry. $^{**}p < 0.01$ versus shSIK2 [ATRA], $^{\#\#}p < 0.01$ versus shSIK3 [ATRA]

suggesting that kinases activities may not be necessary for differentiation. Intriguingly, we found that co-treatment of ATRA and SIK inhibition caused synergistic inhibition of GSK-3β activity as evidenced by enhanced expression levels of p-GSK-3β on Ser9, which can be phosphorylated by Akt leading to GSK-3β inactivation [22, 23]. Of note, GSK-3 inhibition has been reported to synergize with ATRA in the induction of differentiation of AML cells [24, 25]. In this context, it is reasonable to assume that the inactivation of GSK-3β by Akt pathway may contribute to the differentiation effects of ATRA in combination with SIK inhibition. To clarify this, further rescue experiments performed using the cells expressing an Akt unresponsive GSK-3β mutant are essential.

In summary, we provide evidence showing enhanced AML cell differentiation upon with ATRA in combination with SIK inhibition. The combination of ATRA and SIK inhibition synergistically induces differentiation, at least partly through Akt activation. Although this combination therapy requires further clinical and mechanistic studies, the data presented here suggest that ATRA coupled with SIK inhibition may lead to new applications of differentiation-based approaches for AML and other leukemias.

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Compliance with ethical standards

Conflict of interest None declared.

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