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ORIGINAL ARTICLE

Sensitivity of *MLL*-rearranged AML cells to all-*trans* retinoic acid is associated with the level of H3K4me2 in the *RARα* promoter region

K Sakamoto, T Imamura, M Yano, H Yoshida, A Fujiki, Y Hirashima and H Hosoi

All-*trans* retinoic acid (ATRA) is well established as differentiation therapy for acute promyelocytic leukemia (APL) in which the PML–RARα (promyelocytic leukemia-retinoic acid receptor α) fusion protein causes blockade of the retinoic acid (RA) pathway; however, in types of acute myeloid leukemia (AML) other than APL, the mechanism of RA pathway inactivation is not fully understood. This study revealed the potential mechanism of high ATRA sensitivity of mixed-lineage leukemia (*MLL*)-*AF9*-positive AML compared with *MLL-AF4/5q31*-positive AML. Treatment with ATRA induced significant myeloid differentiation accompanied by upregulation of RARα, C/EBPα, C/EBPε and PU.1 in *MLL-AF9*-positive but not in *MLL-AF4/5q31*-positive cells. Combining ATRA with cytarabine had a synergistic antileukemic effect in *MLL-AF9*-positive cells *in vitro*. The level of dimethyl histone H3 lysine 4 (H3K4me2) in the *RARα* gene-promoter region, *PU.1* upstream regulatory region (URE) and *RUNX1* + 24/ + 25 intronic enhancer was higher in *MLL-AF9*-positive cells than in *MLL-AF4*-positive cells, and inhibiting lysine-specific demethylase 1, which acts as a histone demethylase inhibitor, reactivated ATRA sensitivity in *MLL-AF4*-positive cells. These findings suggest that the level of H3K4me2 in the *RARα* gene-promoter region, *PU.1* URE and *RUNX1* intronic enhancer is determined by the *MLL*-fusion partner. Our findings provide insight into the mechanisms of ATRA sensitivity in AML and novel treatment strategies for ATRA-resistant AML.

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INTRODUCTION

Differentiation therapy using all-*trans* retinoic acid (ATRA) is well established for the treatment of acute promyelocytic leukemia (APL)[1,2] in which the PML–RARα (promyelocytic leukemia-retinoic acid receptor α) fusion protein causes blockade of the retinoic acid (RA) pathway.[3,4] There has been significant success using ATRA to treat APL, but there has been little research into the use of ATRA in a non-APL acute myeloid leukemia (AML) setting. In addition, ATRA does not induce myeloid differentiation in non-APL AML cells. Myeloid differentiation is regulated via the activation of *RARα* gene by ATRA.[4,5] Studies of the molecular mechanism blocking differentiation in non-APL AML cells indicate that expression of RARα is diminished in non-APL AML cells and that its restoration can induce differentiation of human AML cells.[6,7] These observations suggest that a lack of RARα expression impairs the RA pathway in non-APL AML cells, blocking myeloid differentiation. In addition, these studies also revealed that epigenetic mechanisms, including DNA methylation, histone methylation and acetylation, are involved in the repression of RARα expression in non-APL AML cells.[6,7] Based on these findings, recent studies have focused on the development of differentiation therapies for non-APL AML using ATRA combined with epigenetic modifiers.[8–12] Recently, it has been reported that inhibition of lysine-specific demethylase 1 (LSD1), which demethylates dimethyl histone 3 lysine 4 (H3K4me2) to silence expression of its target gene, reactivates the RA pathway in non-APL AML.[13,14]

Rearrangements of the mixed-lineage leukemia (*MLL*) gene, which lies on chromosome 11, can involve any of the 79 potential translocation partner genes.[15] *MLL*-rearranged leukemia is a heterogeneous group, and clinical outcome depends on the translocation partner gene involved.[16,17] AML patients with t(9;11) and t(11;19) translocations are classified as intermediate risk, while those with all other *MLL*-fusion partners are classified as high risk.[18] In addition, patients with *MLL*-rearranged AML grouped according to the translocation partner gene involved can be further stratified by the gene expression signature and promoter methylation pattern of their leukemic cells.[19,20] In terms of induction of myeloid differentiation in *MLL*-rearranged AML, we have previously described that the demethylating agent 5-aza-2′-deoxycitidine enhanced the sensitivity of *MLL-AF9*-expressing AML cells to ATRA,[8] and Iijima *et al.*[9] have also reported that the combination of the histone deacetylase inhibitor trichostatin A and ATRA was effective as a growth inhibitor and differentiation enhancer in *MLL-AF9*-expressing leukemia cells. In addition, Niitsu *et al.*[21] demonstrated that ATRA sensitivity of *MLL*-rearranged AML cells were varied and that might be related to *p16* expression level. These findings suggest that chromatin remodeling, such as histone modification by methylation or acetylation, is an important factor for ATRA sensitivity in *MLL*-rearranged AML, and the epigenetic priming of RA pathway is necessary for the differentiation therapy in *MLL*-rearranged AML.

Herein, to determine whether the different partner gene translocations of *MLL*-rearranged AML affect the sensitivity of ATRA, we investigated the relationship between the level of H3K4me2 in the *RARα* gene-promoter region, *PU.1* gene upstream regulatory region (URE) and *RUNX1* gene intronic enhancer and sensitivity to ATRA, and whether, by inhibiting H3K4me2 demethylation in ATRA-resistant cells, sensitivity could be induced in MLL-rearranged human AML cells and murine immortalized cells expressing *MLL-AF9* or *MLL-AF5q31*.

Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, Japan. Correspondence: Dr T Imamura, Department of Pediatrics, Kyoto Prefectural University of Medicine, Kajii-cho Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan.
E-mail: imamura@koto.kpu-m.ac.jp
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MATERIALS AND METHODS

Cell lines and culture

Human AML cell lines, THP-1 (American Type Culture Collection, Manassas, VA, USA) and MOLM-13 (kindly provided by Dr Bert A. van der Reijden, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands) bearing the *MLL-AF9* gene fusion and KOCL-48 (kindly provided by Dr Kanji Sugita, Yamanashi University, Kofu, Japan) bearing *MLL-AF4* were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (10 mg/ml) at 37 °C in a 5% CO_2 humidified atmosphere.

Reagents

ATRA (Sigma-Aldrich, St. Louis, MO, USA), dissolved in dimethyl sulfoxide, and cytarabine (Sigma-Aldrich), dissolved in water, were stored as 1 mM stock solutions in small aliquots at − 20 °C. Tranylcypromine (TCP), a nonreversible LSD1 inhibitor purchased from Sigma-Aldrich, was prepared as a 10 mM stock solution in dissolved dimethyl sulfoxide and stored at − 20 °C.

Retroviral constructs and transduction of Lin$^-$ murine hematopoietic progenitors

Retroviral constructs encoding either human *MLL-AF9* or *MLL-AF5q31* fusion genes were used to establish murine cell lines expressing MLL-AF9 or MLL-AF5q31 fusion proteins as described previously.[8]

Clinical AML cell samples

Primary leukemic cells expressing the *MLL-AF9* fusion were obtained from the diagnostic bone marrow samples of two pediatric AML patients following informed parental consent, in accordance with the revised Helsinki protocol. The AML cells were purified from bone marrow as mononuclear cells using Ficoll density-gradient centrifugation and stored in liquid nitrogen. On thawing, cells were plated in methylcellulose medium (MethoCult H4434, Stem Cell Technologies, Vancouver, BC, Canada) at 1.0×10^6/ml and cultured with ATRA (1 μM).

Morphological studies

Cells at 1.0×10^6/ml were cultured with ATRA (1 μM) and/or TCP (10 μM) for 3 days. Cytospin preparations were stained with May-Grünwald Giemsa.

Nitroblue tetrazolium (NBT) reduction test for detection of myeloid differentiation

After culture with ATRA (1 μM) and/or TCP (10 μM) for 3 days, cells were subjected to a NBT reduction test using the NBT Reduction Kit (Sigma-Aldrich) according to the manufacturer's instructions. The percentage of cells containing the precipitated formazan particles was determined by light microscopy. At least 200 cells were counted per sample.

Cytotoxicity assay

ATRA, TCP and cytarabine cytotoxicity were measured by cell viability using a WST assay (Cell Count Reagent SF, Nacalai Tesque Inc., Kyoto, Japan). The concentration of each drug causing 50% growth inhibition (IC50) was determined. The effect of ATRA and cytarabine and of ATRA and TCP cytotoxic interactions were determined from the combination index (CI); $CI < 1$, $CI = 1$ and $CI > 1$ indicate a synergistic, an additive and an antagonistic effect, respectively. The CI was calculated using the following equation: $CI = ((D)1/(Dx)1) + ((D)2/(Dx)2)$. (Dx)1 and (Dx)2 are concentrations of each drug when each drug inhibits cell proliferation at the 50% growth level individually. (D)1 and (D)2 are concentrations of each drug when combination treatment of two drugs inhibit cell proliferation at the 50% growth level.

Flow cytometric analysis

Cells (1.0×10^6) were harvested, washed twice with PBS and incubated for 30 min with phycoerythrin-conjugated anti-human and anti-mouse Mac-1 (murine CD11b) antibody (BD Biosciences, Sparks, MD, USA) and analyzed on a FACS Calibur (BD Biosciences) with the FlowJo software (Treestar, San Carlos, CA, USA).

Cell-cycle analysis

Cells were harvested, washed twice with PBS and incubated for 30 min with propidium iodide (PI) to stain DNA. Propidium iodide fluorescence was analyzed using a FACS Calibur, and the cell-cycle phase was determined on the basis of DNA content using the ModFit *LT* software (Verity Software House, http://www.vsh.com/).

Real-time reverse transcriptase–PCR (RT-PCR)

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Venio, The Netherlands) according to the manufacturer's instructions. The SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA according to the manufacturer's instructions, and real-time RT-PCR was performed using the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green 1 Master Mix (Takara Bio, Tokyo, Japan). Relative expression of target mRNA was determined using the comparative threshold (ΔCt) method. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The primer pairs used in this study are listed in Supplementary Table S1. A standard curve analysis with stepwise sample dilution demonstrated that all primer pairs had similar efficiency (data not shown).

Immunoblot analysis

Immunoblot analysis was performed as described previously.[22] The following primary antibodies were used: anti-RARα (diluted 1:200), anti-C/EBPα (diluted 1:750), anti-C/EBPε (diluted 1:200) and anti-PU.1 (diluted 1:500). All antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology Inc., Danvers, MA, USA) according to the manufacturer's instructions. IP was performed with the following antibodies: anti-H3K4me2 (Abcam, Tokyo, Japan) and anti-histone H3 (Cell Signaling Technology Inc.). IP DNA in each IP sample of human cell lines was analyzed by quantitative RT-PCR using *RARα* gene-specific primers covering the promoter and 5′ untranslated region (5′UTR): from − 1000 to + 1000 bp from the transcriptional start site,[23,24] *PU.1* URE[25] and *RUNX1* + 24/ + 25 intronic enhancer.[26] Also, IP DNA in each IP sample of murine cell lines was analyzed by quantitative RT-PCR using *Rarα* gene-specific primers covering the promoter and 5′UTR:from − 1000 to + 1000 bp from the transcriptional start site, *Sfpi.1* URE and *Runx1* + 24/ + 25 intronic enhancer.[25,26] The list of primer sequences are shown in Supplementary Tables 2 and 3. All expression values were normalized against histone H3.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

ATRA induces myeloid differentiation and growth inhibition with cell-cycle arrest in human *MLL-AF9*-positive AML cells

First, we evaluated whether ATRA could induce myeloid differentiation, as observed in APL, in three human AML cell lines with *MLL* rearrangements. ATRA induced morphological changes, such as irregularly shaped nuclei, extended cytoplasm and the appearance of fine granules, more markedly in THP-1 and MOLM-13 cell lines, which bear the *MLL-AF9* fusion gene, than in KOCL-48 which bears *MLL-AF4* (Figure 1a). These morphological changes corresponded with a reduction in NBT, which indicated an increase in mature myeloid cell function (for example, phagocytosis activity), and the induction of CD11b on flow cytometric analysis (Figures 1b and c). Furthermore, ATRA induced G0/G1 arrest more clearly in the *MLL-AF9*-positive cell lines than in the *MLL-AF4*-positive cells and had a much lower IC50 with the *MLL-AF9*-positive cell lines (THP-1 and MOLM-13) than with the *MLL-AF4*- (KOCL-48) positive cells (3.91 ± 0.87 and 1.24 ± 0.70 vs 77.2 ± 7.37 μM; Supplementary Figure S1a and Figure 1d). Taken together, these data demonstrate that the effects of ATRA on the induction of myeloid differentiation were more apparent, and that the effects of ATRA on growth inhibition were significantly greater, on the *MLL-AF9*-positive AML cell line than

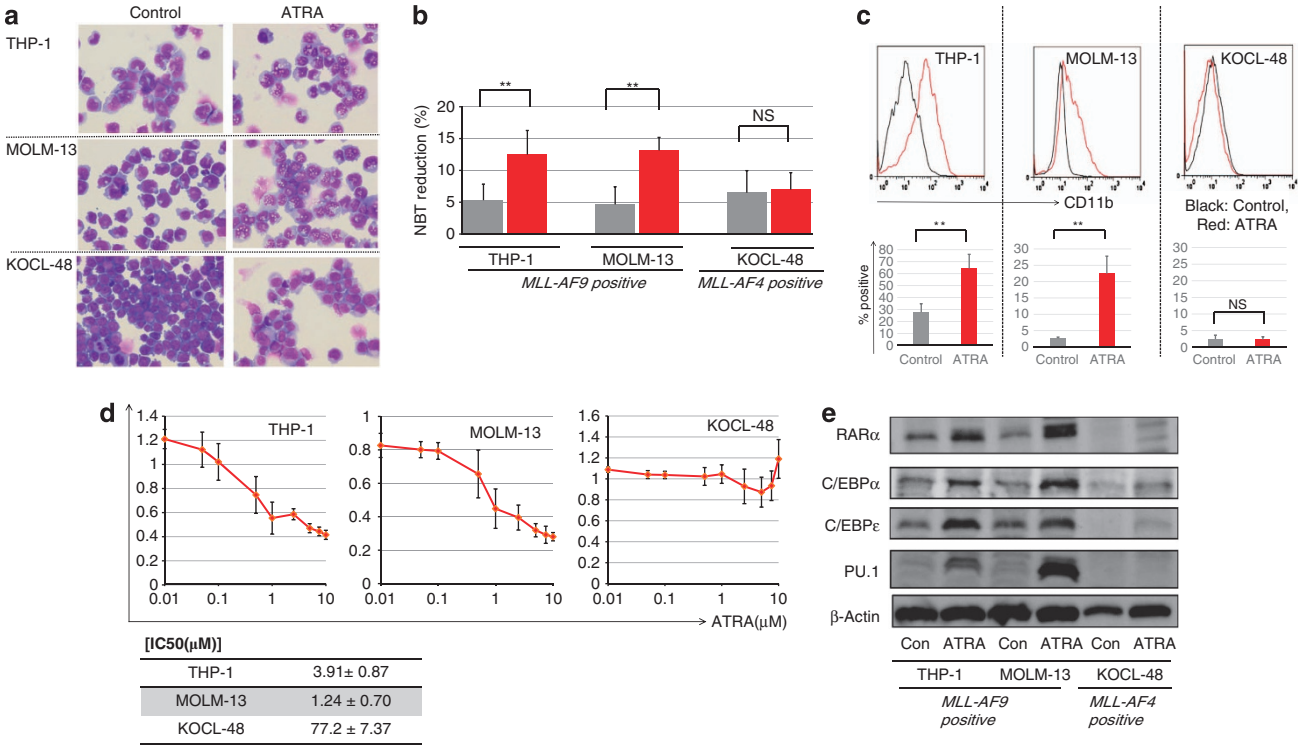


Figure 1. The effect of ATRA on human *MLL*-rearranged AML cell lines. (**a**) Photomicrographs of THP-1, MOLM-13, and KOCL-48 cell lines following incubation with ATRA (1 μM) for 72 h. Cytospin preparations were stained with May-Grünwald Giemsa. (**b**) Effect of ATRA on a reduction in NBT. Cells were incubated with ATRA for 72 h. Results represent the means ± s.d. of three independent experiments. $^{**}P < 0.05$; NS, not significant. (**c**) CD11b expression determined by FACS analysis following incubation with ATRA (1 μM) for 72 h. Black line, untreated cells; red line, cells treated with ATRA. Representative results are shown, and the bars represent the means ± s.d. of three independent experiments. $^{**}P < 0.05$; NS, not significant. (**d**) Cytotoxicity of ATRA on THP-1, MOLM-13 and KOCL-48 cell lines. The number of viable cells was assessed following incubation with titrating doses of ATRA (range, 0–10 μM) for 96 h. ATRA IC50 values were determined for each cell line. (**e**) Expression of RARα, C/EBPα, C/EBPε and PU.1 determined by immunoblot analysis after incubation with ATRA (1 μM) for 72 h. β-Actin was used as a control. Representative results from three independent experiments are shown.

on the *MLL-AF4*-positive cell line. These data were consistent with the previous studies showing *MLL-AF9*-positive AML cell line was sensitive to ATRA.[8,9]

The RA pathway is more profoundly impaired in human *MLL-AF4*-positive cells than in *MLL-AF9*-positive cells

The expression of *RARα*, *C/EBPα*, *C/EBPε* and *PU.1* genes is modulated in myeloid differentiation by RA. Using western blotting analysis, we found that RARα and C/EBPε expression levels, which are directly regulated by ATRA, were increased in response to ATRA in the *MLL-AF9*-positive cell lines THP-1 and MOLM-13 but not in the *MLL-AF4*-positive cell line KOCL-48. In addition, expression of C/EBPα and PU.1, which are important transcriptional factors in myeloid differentiation, was also increased only in *MLL-AF9*-positive cells (Figure 1e).

The effect of ATRA on primary *MLL-AF9*-positive AML cells

To confirm these effects of ATRA on myeloid differentiation, we evaluated the expression levels of *RARα*, *C/EBPα*, *C/EBPε* and *PU.1* in two primary AML samples bearing *MLL-AF9* fusion using real-time RT-PCR analysis. In accordance with the results of the western blotting analysis of human *MLL-AF9*-positive AML cell lines, the expression levels of *RARα*, *C/EBPα*, *C/EBPε* and *PU.1* were upregulated in both primary *MLL-AF9*-positive AML samples (Supplementary Figure S2). Collectively, these results showed clearly that ATRA was able to induce myeloid differentiation and activate the RA pathway in *MLL-AF9*-positive AML cells. In contrast, ATRA did not induce myeloid differentiation or activate the RA pathway in *MLL-AF4*-positive cells.

The effect of ATRA on murine *MLL*-rearranged immortalized cells

To confirm that the particular *MLL*-fusion partner (*AF9* or *AF4*) directly determined the activity of the RA pathway, our aim was to generate immortalized murine cells expressing *MLL-AF9* or *MLL-AF4*; however, we were unable to transform murine hematopoietic progenitor cells using *MLL-AF4*. Therefore *AF5q31*, a member of the *AF4* family of genes, fused with *MLL* was used as an alternative.[8] Similar to the results of the experiments with human AML cells, ATRA induced morphological changes only in the murine *MLL-AF9*-expressing cells (Figure 2a), and these changes were accompanied by a reduction in NBT and upregulation of Mac 1expression (Figures 2b and c). In addition to these effects, the percentage of cells in G0/G1 was greater among the *MLL-AF9*-expressing cells (Supplementary Figure S1b), and the ATRA IC50 was much lower for *MLL-AF9*-expressing cells than for *MLL-AF5q31*-expressing cells (2.01 ± 0.39 vs 32.6 ± 14.5 μM; Figure 2d). The expression levels of *Rarα*, *C/ebpα*, *C/ebpε* and *Sfpi.1* determined by real-time RT-PCR were significantly upregulated in response to ATRA in the *MLL-AF9*-expressing cells but were unchanged in the *MLL-AF5q31*-expressing cells (Figure 2e), suggesting that murine Lin^- hematopoietic progenitor cells expressing *MLL-AF9*, but not those expressing *MLL-AF5q31*, remain sensitive to ATRA. Thus, it could be concluded that particular *MLL*-fusion partners affect sensitivity to ATRA.

A synergistic antileukemic effect between cytarabine and ATRA in *MLL-AF9*-positive AML cells

To explore the possibility of a synergistic antileukemic effect between ATRA and cytarabine, one of the major drugs used to

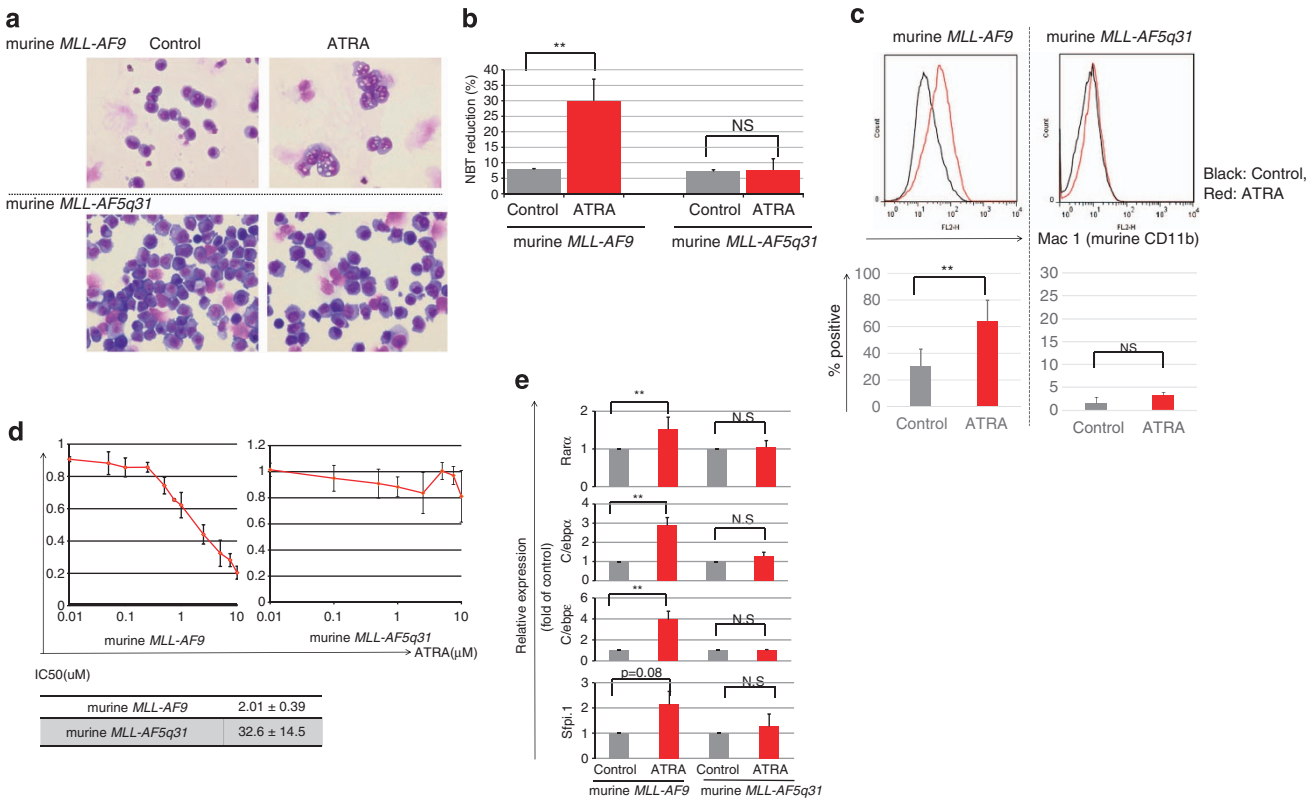


Figure 2. The effect of ATRA on murine *MLL*-rearranged immortalized cells. (**a**) Photomicrographs of murine immortalized cell lines expressing *MLL-AF9* and *MLL-AF5q31* following incubation with ATRA (1 μM) for 72 h. Cytospin preparations were stained with May-Grünwald Giemsa. Representative results from three independent experiments are shown. (**b**) Effect of ATRA on reduction in NBT. Murine immortalized cell lines were each cultured with ATRA (1 μM) for 72 h. Results represent the mean ± s.d. of three independent experiments. $^{**}P<0.05$; NS, not significant. (**c**) Mac 1 expression determined by FACS analysis following incubation for 72 h with ATRA (1 μM). Black line, untreated cells; red line, cells treated with ATRA. Representative results are shown, and the bars represent the means ± s.d. of three independent experiments. $^{**}P<0.05$; NS, not significant. (**d**) Cytotoxicity of ATRA on *MLL*-rearranged murine immortalized cell lines. The number of viable cells was assessed following incubation with titrating doses of ATRA (range, 0–10 μM) for 72 h. ATRA IC50 values were determined for each cell line. (**e**) Expression of *Rarα*, *C/ebpα*, *C/epbε and Sfpi.1* determined by real-time RT-PCR. Cells were harvested after incubation with ATRA (1 μM) for 72 h. Results represent the mean ± s.d. of three independent experiments. $^{**}P<0.05$; NS, not significant.

treat AML, we treated human and murine *MLL-AF9*-positive cells (THP-1 and MOLM-13 human AML cell lines and the immortalized murine *MLL-AF9*-expressing cells) with a titrating dose of cytarabine in combination with 1 μM ATRA or without ATRA. We found that the presence of ATRA decreased the cytarabine IC50 in THP-1 and MOLM-13 cell lines and in the murine *MLL-AF9*-expressing cells (cytarabine IC50 of 3.69 ± 1.00, 0.042 ± 0.030 and 0.060 ± 0.012 μM, respectively, without ATRA, vs 0.17 ± 0.075, 0.0038 ± 0.0031 and 0.015 ± 0.0026 μM, respectively, with ATRA). The combined effects of cytarabine and ATRA on cell-growth inhibition were clearly synergistic in the three cell lines (Figures 3a and b). ATRA in combination with cytarabine was, therefore, more effective than cytarabine alone for ablating *MLL-AF9*-positive cells *in vitro*.

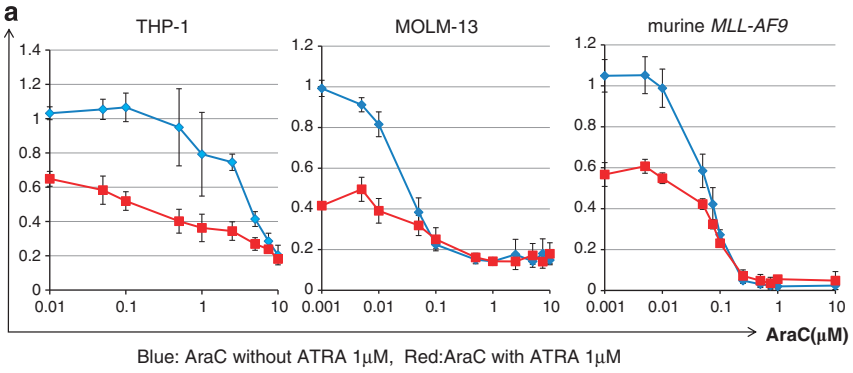
ChIP assays of H3K4me2 on *MLL*-rearranged human AML cell lines and murine *MLL*-rearranged immortalized cells

Next, we carried out ChIP assays on *MLL*-rearranged human AML cell lines to investigate whether inactivation of the RA pathway was correlated with decreased H3K4me2 in the *RARα*-promoter region, which contained the RA response elements.[23] IP DNA was analyzed using *RARα* gene-specific primers mapping to the promoter and 5′UTR from −1000 to +1000 bp from the transcriptional start site. The levels of H3K4me2 normalized against histone H3 were lower in the *MLL-AF4*-positive cell line, KOCL-48, than in the *MLL-AF9*-positive cell lines, THP-1 and MOLM-13. These findings revealed that ATRA sensitivity correlates with the H3K4me2 level in the *RARα* gene in *MLL*-rearranged AML cell lines (Figure 4a). In addition, we evaluated the histone modification status at the *PU.1* proximal and distal URE and *RUNX1* + 24/ + 25 intronic enhancer, which were also associated with myeloid differentiation.[25,26] The levels of H3K4me2 at these regions were lower in the *MLL-AF4*-positive cell line, KOCL-48, than in the *MLL-AF9*-positive cell lines, THP-1 and MOLM-13 (Figure 4b). Collectively, *MLL-AF9*-positive cell lines bear higher H3K4me2 level in the *RARα* gene, *PU.1* URE and *RUNX1* intronic enhancer than *MLL-AF4* positive cell line, resulting in efficient induction of myeloid differentiation in *MLL-AF9*-positive cell lines by ATRA.

To confirm the particular *MLL*-fusion partner (*AF9* or *AF4*) directly modified the histone modification, such as the H3K4me2 level, we investigated the levels of H3K4me2 at *Rarα* gene-promoter, *Sfpi.1* URE, and *Runx1* intronic enhancer in immortalized murine cells expressing *MLL-AF9* or *MLL-AF5q31*. Consistent with the result of human AML cell lines, the levels of H3K4me2 normalized against histone H3 were lower in the murine cells expressing *MLL-AF5q31* than in the murine cells expressing *MLL-AF9* at *Rarα* gene-promoter, *Sfpi.1* URE, and *Runx1* intronic enhancer (Figures 4c and d).

TCP restores ATRA sensitivity in the *MLL-AF4*-positive and ATRA-resistant cell line, KOCL-48

LSD1 demethylates H3K4me2, silencing the target gene; inhibition of LSD1 leads to an increase in H3K4me2. To determine whether the H3K4me2 status of the *RARα* promoter region and 5′UTR affected the RA pathway, KOCL-48 cells were treated with TCP, an



b

IC50(uM)	AraC without ATRA 1μM	AraC with ATRA 1μM	Combination Index
THP-1	3.69±1.00	0.17±0.075	0.23
MOLM-13	0.042±0.030	0.0038±0.0031	0.46
murine *MLL-AF9*	0.060±0.012	0.015±0.0026	0.82

Figure 3. Synergistic cytotoxic effects between ATRA and cytarabine in *MLL*-AF9-positive cells. (**a**) THP-1, MOLM-13 and murine *MLL-AF9*-expressing immortalized cells were cultured with a range of concentrations of cytarabine (0–10 μM) with 1 μM ATRA or without. After incubation for 72 h in MLL-AF9-expressing immortalized cells and 96 h in human cell lines (THP-1 and MOLM-13), the number of viable cells was assessed by a WST assay. (**b**) Cytarabine IC50 with or without 1 μM ATRA values were determined for each cell line.

inhibitor of LSD1. A combination of 1 μM ATRA and 10 μM TCP (ATRA/TCP) induced more marked morphological changes and caused a bigger reduction in NBT than either ATRA or TCP alone (Figures 5a and b). Furthermore, ATRA/TCP induced higher-intensity expression of CD11b and increased the proportion in G0/G1 cell-cycle arrest (Figure 5c and Supplementary Figure S1c). Western blotting analysis revealed that the expression of RARα, C/EBPα, C/EBPε and PU.1 increased in response to ATRA/TCP (Figure 5d), suggesting that the combination of ATRA and TCP reactivates the RA pathway. We therefore treated KOCL-48 cells with a titrating dose of ATRA with 10 μM TCP or without TCP. The ATRA IC50 was significantly decreased when added in combination with TCP, and the CI showed that ATRA and TCP had a synergistic effect (Figure 5e, ATRA IC50 of 72.2 ± 7.95 μM without TCP vs 12.9 ± 3.04 μM with TCP). Finally, we tested whether TCP could upregulate the level of H3K4me2 in the *RARα* gene-promoter region and 5′UTR. The levels of H3K4me2 in the presence of TCP were increased in a dose-dependent manner (Figure 5f). Similarly, the level of H3K4me2 in the *PU.1* URE and *RUNX1* intronic enhancer were also upregulated in the presence of TCP (Figure 5g). These findings suggest that inactivation of the RA pathway and myeloid differentiation block are induced by a decrease in the level of H3K4me2 in the *RARα* gene-promoter, the *PU.1* URE and *RUNX1* intronic enhancer.

DISCUSSION

In this study, we have examined the mechanisms of ATRA sensitivity in non-APL AML cells. We found that *MLL-AF9*-positive cells were sensitive to ATRA and that this was accompanied by the upregulation of myeloid differentiation genes, such as *C/EBPα*,[27–29] *C/EBPε*[30–32] and PU.1.[33,34] In contrast, *MLL-AF4/AF5q31*-positive cells were not sensitive to ATRA, suggesting that the RA pathway is more strongly inactivated in *MLL-AF4/AF5q31*-positive cells than in *MLL-AF9*-positive cells. In addition, the experiments on murine *MLL*-rearranged immortalized cells revealed that the response to ATRA was dependent on the *MLL*-fusion partner gene.

Furthermore, we demonstrated a synergistic antileukemic effect between cytarabine and ATRA in *MLL-AF9*-positive cells, which were sensitive to ATRA, *in vitro*. Previous studies have shown that ATRA induces cell-cycle arrest and apoptosis in *nucleophosmin 1* (*NPM1*)-mutated AML cells and that the *NPM1* mutation sensitizes the AML cells to ATRA and cytarabine *in vitro*.[35,36] However, several subsequent studies have failed to reveal any clinical benefit of combining ATRA with conventional chemotherapy.[37–39] Thus it will be important to determine whether the synergistic antileukemic effect of cytarabine and ATRA seen in *MLL-AF9*-positive AML cells *in vitro* is corroborated *in vivo*.

We also demonstrated that the level of H3K4me2 in the *RARα* gene-promoter region was closely associated with ATRA sensitivity. Our results showed that loss of H3K4me2 levels in *RARα* resulted in the inactivation of the RA pathway and that inhibition of *LSD1* could reactivate the RA pathway in ATRA-resistant *MLL-AF4*-positive cells. In addition, we revealed that the levels of H3K4me2 in the *PU.1* URE and *RUNX1* intronic enhancer were also associated with the degree of ATRA-mediated myeloid differentiation, which was restored by the inhibition of LSD1.

Recent studies demonstrated that MLL was directly bound to RUNX1 physically and functionally.[40,41] Also, RUNX1 bound to the *PU.1* URE, which has enhancer and repressor activity to regulate *PU.1* expression.[24,42] Furthermore, *RUNX1* + 24/ + 25 intronic enhancer was identified as a *cis*-regulatory element of *RUNX1* gene expression in hematopoietic stem cells.[25] Collectively, MLL, RUNX1 and PU.1 were closely interacted, and these interactions were modified by epigenetic regulation at the gene regulatory region, such as *PU.1* URE or *RUNX1* intronic enhancer, suggesting MLL fusions might interfere the myeloid differentiation caused by RUNX1 and PU.1 in different manner. Epigenetic research in leukemia is a rapidly developing field[43] and in APL the *PML–RARα* fusion gene is now known to recruit various histone-modifying enzymes. Binding of the large PML–RARα fusion-protein complex to the promoter of the *RARα* target gene 'closes' the chromatin structure, resulting in the repression of gene expression, blocking the RA pathway, and thereby allowing oncogenic programs to progress.[44,45] Similarly, the AML1–ETO fusion protein (produced by the t(8;21) translocation) also recruits histone deacetylase and DNA methyltransferase, which block the RA pathway and promote

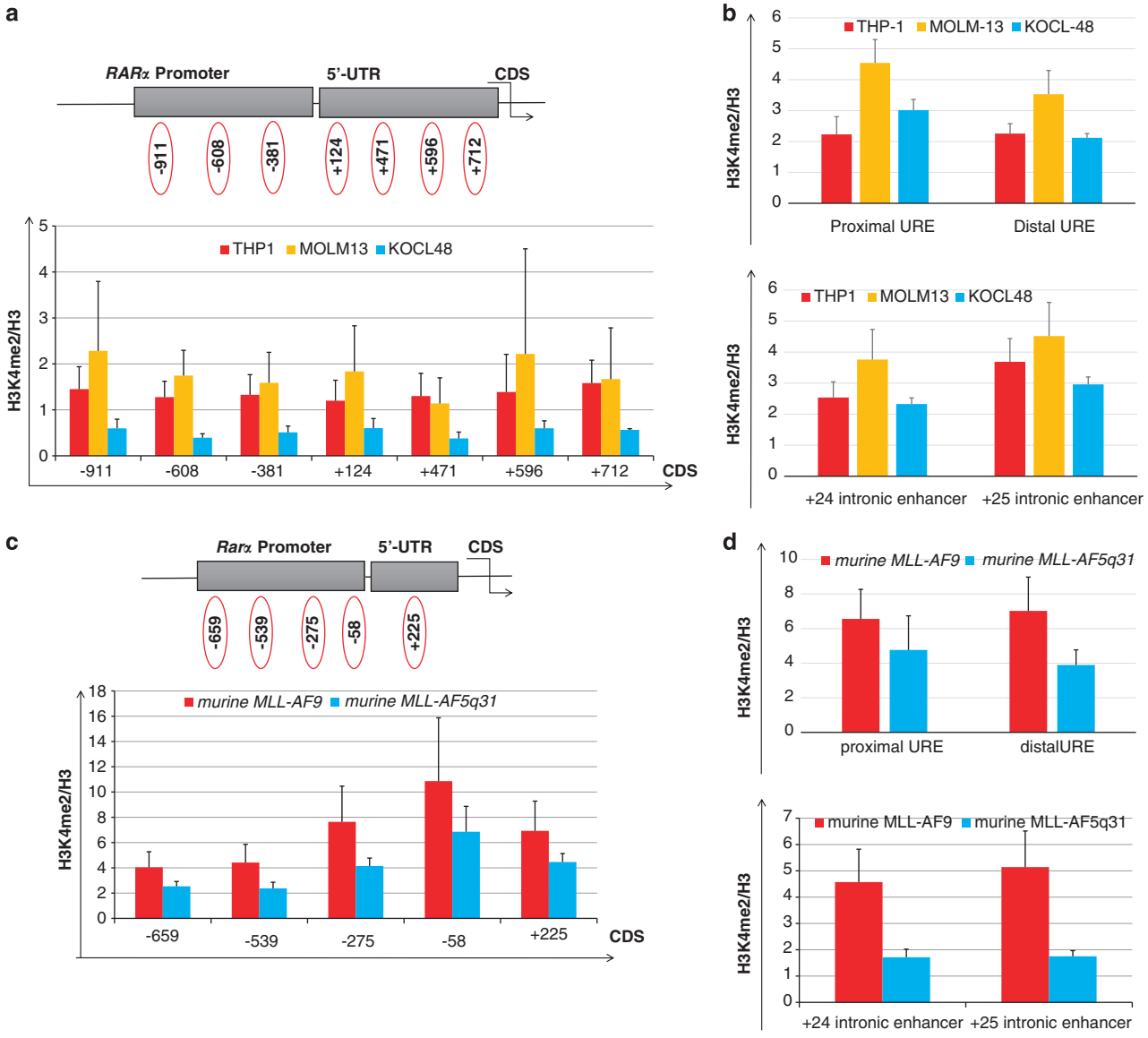


Figure 4. H3K4me2 modification of the *RARα*, *Rarαgene*, *PU.1/Sfpi.1* URE and *RUNX1/Runx1* intronic enhancer in human AML cell lines and murine immortalized cell lines. After cells were incubated with ATRA (1 μM) for 72 h, ChIP assays were performed using antibodies to H3K4me2 and histone H3 (H3). Precipitated DNA was analyzed by real-time RT-PCR using primers mapping to within the promoter region and 5′UTR) of the *RARα* gene (**a**) and *PU.1* URE or *RUNX1* + 24/ + 25 intronic enhancer (**b**), the promoter region and 5′UTR of the *Rarα* gene (**c**) and *Sfpi.1* URE or *Runx1* + 24/ + 25 intronic enhancer (**d**). Results represent the mean ± s.d. of two independent ChIP experiments and a total of four independent PCR analysis.

leukomogenesis.[46,47] In addition, the demethylating agent or histone deacetylase inhibitor enhance the ATRA-mediated myeloid differentiation in *MLL-AF9*-expressing AML cells.[8,9] These observations suggest that epigenetic modification is an important factor for leukomogenesis in some AML subtypes, including *MLL*-rearranged AML.

LSD1, which demethylates histone H3 at lysine 4 (H3K4) and lysine 9 (H3K9), was the first histone demethylase to be identified.[48] LSD1 is overexpressed in various cancers, and recent researches showed that this epigenetic modifier is associated with the development of drug resistance.[49,50] Schenk *et al.*[13] reported that the LSD1 inhibitor TCP can reactivate ATRA sensitivity in the ATRA-resistant TEX cell line *in vitro* and diminish the engraftment of primary AML samples *in vivo*. In addition, they demonstrated that the combination of ATRA and TCP led to upregulation of gene expression associated with the myeloid differentiation program and apoptosis, accompanied by increased amounts of H3K4me2 near the transcriptional start sites.[13] Similarly, in the current study we found that the amount of H3K4me2 in the region from − 1000 to + 1000 from the transcriptional start site of *RARα* was correlated with ATRA sensitivity and that TCP could reactivate ATRA sensitivity in an ATRA-resistant cell line expressing the MLL-AF4 fusion protein. We also revealed that TCP restored the levels of H3K4me2 in the *PU.1* URE and *RUNX1* intronic enhancer in the ATRA-resistant cell line. The IC50 of ATRA in combination with TCP was, however, much higher than the pharmacological concentration of ATRA for clinical use, suggesting that a more potent inhibitor of histone demethylase is required for reactivation of ATRA sensitivity in an ATRA-resistant cell.[51–53]

In conclusion, we found that *MLL-AF9*-positive AML cells were sensitive to ATRA and that a high level of H3K4me2 in the promoter region of the *RARα* gene, *PU.1* URE and *RUNX1* intronic enhancer is associated with ATRA sensitivity in this subtype.

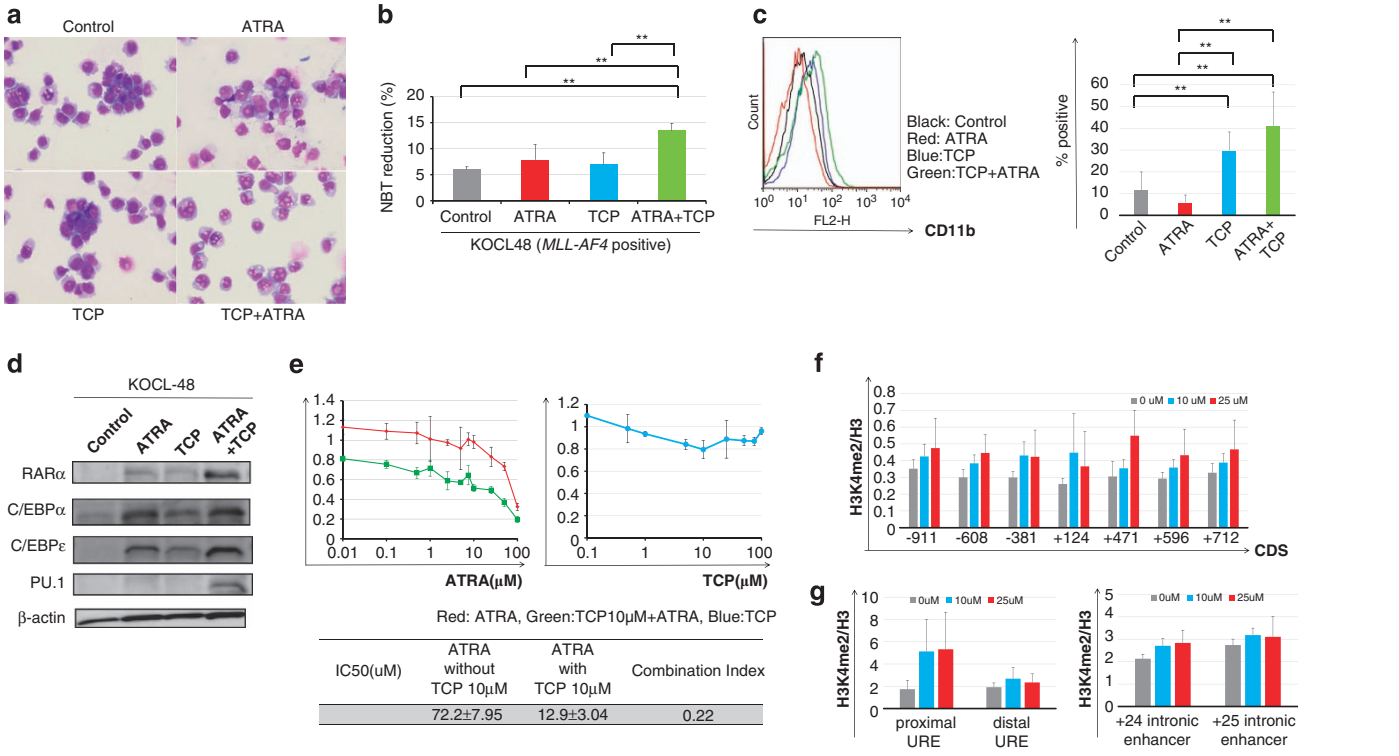


Figure 5. ATRA and TCP can reactivate the RA pathway in the *MLL-AF4*-positive AML cell line, KOCL-48. (**a**) Photomicrographs of KOCL-48 cells untreated and treated with ATRA (1 μM) and/or TCP (10 μM). Cytospin preparations were stained with May-Grünwald Giemsa. Representative results from three independent experiments are shown. (**b**) Effect of ATRA/TCP on reduction in NBT. KOCL-48 cells were cultured with ATRA/TCP for 72 h. Results represent the mean ± s.d. of three independent experiments. $^{**}P<0.05$; NS, not significant. (**c**) CD11b expression determined by FACS analysis following incubation with ATRA/TCP for 72 h. Black line, untreated cells; red line, ATRA; blue line, TCP; green line, ATRA/TCP. Representative results are shown, and the bars indicate the means ± s.d. of three independent experiments. $^{**}P<0.05$; NS, not significant. (**d**) Expression of RARα, C/EBPα, C/EBPε and PU.1 determined by immunoblot analysis after incubation with ATRA/TCP. β-Actin was used as a control. Representative results from three independent experiments are shown. (**e**) ATRA IC50 values with or without 10 μM TCP in KOCL-48 cells. (**f**) After KOCL-48 cell lines were incubated with TCP (0, 10, 25 μM) for 72 h, DNA was obtained from the IP chromatin using antibodies to H3K4me2 and histone H3 (H3). Precipitated DNA was analyzed by real-time RT-PCR using primers mapping to within the promoter region and 5′UTR of the *RARα* gene and (**g**) the *PU.1* URE or *RUNX1* + 24/ + 25 intronic enhancer. The results shown are normalized to the level of H3. Results represent the mean ± s.d. of two independent ChIP experiments and a total of four independent PCR analysis.

Addition of ATRA to cytarabine had a synergistic antileukemic effect on *MLL*-AF9-positive AML cells *in vitro*, suggesting that in cases of AML with high levels of H3K4me2 in the *RARα* promoter region, *PU.1* URE and *RUNX1* intronic enhancer, ATRA would be able to sensitize leukemic cells to cytarabine; an *in vivo* study is now required to confirm this. Furthermore, our findings indicate that epigenetic modifiers, such as LSD1 inhibitors, are potentially useful for treating ATRA-resistant AML, including *MLL*-rearranged AML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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