RESEARCH ARTICLE

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Quantitative Profiling of Alpha-Subunit of IL-3 Receptor on Single Acute Myeloid Leukemia Cells by Super-Resolution Imaging

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Abstract

Background: Quantitative profiling of specific cell surface markers is a new approach in characterization of tumor heterogeneity and single cell biology. The current tools have dearth in detection and quantification of receptor proteins on single cells. **Methods:** we focused on our newly developed protocol to determine the distribution pattern and density of cell surface markers on single acute myeloid leukemia cells. Cell surface proteins were labeled with quantum dots (Qdots) followed by super resolution Structured Illumination Microscopy (SIM) imaging to imprisonment the optical signals emitted by Qdots which were further analyzed by software imaris to do three dimensional (3D) structure reconstruction and digital simulation. Furthermore, MTT assays and flow cytometry was performed to establish association between expression of cell surface markers and drug response. **Results:** In the present study, we found that the Molm13 and cytarabine-enriched Molm13 cells exhibit different densities of CD123, an alpha-subunit of interleukin-3 receptor, i.e. 0.92 and 1.73 per μm^2 of cell surface respectively. Sub-populations of Molm13 cells expressing higher densities of CD123 on cells membranes showed resistance against cytarabine. Further study revealed that romidepsin sensitized and augmented the cytotoxicity of cytarabine in Molm13 and cytarabine-enriched Molm13 cells. Romidepsin increased the percentage of cell death-induced by cytarabine from 21.6 % to 28.6 % and 37.1 % to 57.2 % in Molm13 and cytarabine-enriched Molm13 cells respectively. **Conclusion:** Altogether, the study suggests that Molm13 cells have sub-populations with differential expression of CD123+ phenotype. Romidepsin sensitizes and augments the effect of cytarabine in Molm13 and cytarabine-enriched Molm13 cells.

Keywords: Tumor heterogeneity- CD123- Molm13- Romidepsin- Cytarabine- QDot- SIM

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Introduction

Quantitative profiling of cell surface markers is a new approach in characterization of tumor heterogeneity and single cell biology. Differential expression of cell surface markers on different cells makes them an ideal target in tumor targeted therapies (Shi et al., 2019). Several techniques of protein analysis including mass spectrometry, flow cytometry and western blotting have extensively been used to elucidate the role of protein molecules in complex signaling cascades upon interaction with other protein molecules. However, these methods are not useful in describing the distribution pattern of individual protein molecules on single cells within cell population. Single cell mass spectrometry (SCMS) and multi-parameter flow cytometry (MFC) are effective even in infinitesimal amounts of protein analysis but due to limits on fluorescent probes and unsatisfactory resolutions fail to provide single molecule quantitative in situ analysis (Wu and Singh, 2012). Single molecule localization techniques including Stimulated Emission Depletion Microscopy (STED), Photo-activated Localization Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM) can provide the detailed information of protein localization but the requirement of higher intensity illumination, and photo-stable fluorescent probes can damage and alter the specimen. High machine cost and complexity may also inhibit their routine use (Sydor et al., 2015; Tam and Merino, 2015; Feng et al., 2018; Vicidomini et al., 2018; Wang et al., 2019). In the present study we used QDots to label single protein molecules in situ followed by super resolution SIM imaging. QDots emit signals of high brightness and SIM reconstructs the fine structure of cells by computing the interference configurations induced by irradiation with striped-pattern excitation light and provides quantitative information of single molecule (Gustafsson, 2000; Demmerle et al., 2017; Lambert and Waters, 2017). It does not depend on specialized molecular

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staining probes which is a great advantage of SIM over any other super resolution technologies (Xia et al., 2012; Ma et al., 2019; Xi et al., 2019).

Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell disorder in which accumulation of non-functional immature cells, myeloblasts, occurs due to increased proliferation and failure of differentiation in the stem cell compartment. It is the most common form of leukemia seen in adults (Al-Mawali et al., 2017). In case of complete remission, the 5 year overall survival (OS) rate is 35−40% in adults and 60% in children (Tettamanti et al., 2014; Tasian et al., 2017). Without complete remission, the 5 year OS rate is 26%. Only in Europe, the OS rates for men and women are 15 and 18% respectively (Al-Mawali et al., 2017). Targeted therapies provide the opportunities to target several cell surface receptors (Yabushita and Satake, 2018; Assi et al., 2018). Many of the cell surface receptors are equally expressed on normal cells resulting in "on-target off-tumor" effects which challenge the outcomes of targeted therapies. Hence, profiling and analysis of cells surfaces for the identification of novel marker expressed differentially on tumor cells and normal cells is required to cure patients with AML.

CD123, alpha-subunit of IL-3 receptor (IL-3R), is an overexpressed cell surface marker on AML blasts (Jordan et al., 2000). CD123 along with other members of IL-3R system sets the growth, proliferation, differentiation and survival of hematopoietic cell. Literature evidence; Liu et al., (2015) has shown that CD123 is an important marker of leukemia stem cells (LSCs) and is responsible for relapse of the disease by initiating and maintaining the growth of leukemia cells resistant to chemotherapy. In this study, we have shown the differential expression of CD123 on parent and enriched Molm13 AML cells. The sub-population of Molm13 cells with higher densities of CD123+ marker on single cells was increased in cytarabine enriched/resistant cells. Further study revealed that romidepsin sensitized and augmented the effect of cytarabine on both type of cells. Therefore, the study suggests that quantitative profiling data of a specific cell surface marker on single AML cells have potential information to make better treatment strategies for relapsed and chemoresistant AML patients.

Materials and Methods

Antibodies and reagents

Cytarabine, Romidepsin, was purchased from MedChemExpress (USA). Dimethyl sulfoxide (DMSO) was purchased by Sigma Aldrich. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Propidium iodide, Apoptosis kit and BCA protein Assay kit was purchased from Beyotime. Antibodies such as cleaved caspase-3, PARP and GAPDH were purchased from cell signaling technology. Purified anti-human CD123 antibody was purchased from BioLegend. QDot-605 goat F(ab')2 anti-mouse IgG conjugate (H+L) was purchased from invitrogen.

Cell culture

Acute myeloid leukemia Molm13 cell line was purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI1640 supplemented with 15 % FBS, 100 µg/mL streptomycin and 100 units/mL penicillin and maintained at 37°C with 5 % CO_2 in humidified atmosphere and routinely passaged after 48 h.

Enrichment of CD123+ cells

As sub-population of AML cells expressing CD123+ molecules are resistant to cytarabine and result in relapse of AML. Therefore, the sub-population of CD123+ cells was enriched by exposing the Molm13 cells to increasing concentration of cytarabine starting from 1 nM to 5 µM. Enriched population of Molm13 cells was resistant at 5 µM and was coined as Molm13-R5. Molm13-R5 cells were continuously exposed to resistant concentration (5 µM) during experiments.

Cells smear formation and QDot staining

Cultured cells were collected, washed with PBS and fixed with 4 % PFA for 20 minutes at room temperature. After washing three times, 10,000 cells were smeared on glass slides by cytospin4 cytocentrifuge (Thermo Scientific). Cells were blocked with 10 % goat serum in PBS for 1 hour at room temperature followed by overnight incubation with mouse anti-human CD123 monoclonal antibody at 4°C. Subsequently, the cells were washed again and incubated with QDot-conjugated anti-mouse IgG second antibody for 1 hour at room temperature. Finally after washing, the images were captured by SIM (DeltaVision OMX V3, API, USA).

SIM image 3D reconstruction by Imaris

3D structure reconstruction and quantification of CD123 molecules was determined by software imaris as reported by us previously (Xi et al., 2019). Briefly, the raw date of SIM was opened in Bitplane Imaris 7.2.3 followed by brightness/contrast adjustment via show display adjustment mode in edit menu. Camera pointer in navigate mode and mouse wheel was used to rotate the dendrite in 3D and to zoom in/out respectively. Firstly, number of Qdots was counted by clicking Add New Spot icon and in new opened window parameters like Point Style/Quality Window, Sphere, and Radius Scale (0.3) was adjusted. The information of total number of spots was gathered by Statistics icon in Style menu. Secondly, 3D structure reconstruction was reconstructed by Add New Surfaces icon. In new opened window, parameters i.e. Source Chanel window and Smooth were selected and Surfaces Area Detail Level (1µm) was adjusted. Information of newly reconstructed surface was collected by clicking Statistics icon and Detailed window in the Styles menu.

Determination of cell viability

To determine the toxicity of Cytarabine and Romidepsin on Molm13 and Molm13-R5 cells, MTT assay was performed as described previously (Mehmood et al., 2017). Briefly, cells were seeded (10,000/well) in 96-well cell culture plates and treated with different concentrations of cytarabine and romidepsin for 48 h. The cells were then incubated with 10µL MTT (5 mg/mL)

reagent at 37°C for 4 h. Subsequently, 150 µL DMSO was added in each well and cell culture plates were agitated gently to dissolve formazan crystals completely. Cytation 3 cell imaging multi-mode reader (BioTek) was used to measure absorbance at 570 nm. The percentage of cell viability was calculated as follows:

Cell viability (%) = (A570 sample – A570 blank) / (A570 control - A570 blank) × 100

Apoptosis by flow cytometry

Apoptosis effect of cytarabine and romidepsin was determined by Annexin V-FITC/PI double staining apoptosis detection kit (Beyotime, Nanjing, China). Briefly, AML cells were treated with indicated concentrations of cytarabine and romidepsin. Cells were then collected, washed and re-suspended in 400 µl binding buffer. Cells were then incubated with 5 µl Annexin V-FITC for 10 minutes in dark. After that, cells were further incubated with 10 µl PI for another 10 minutes in dark according to manufacturer's instructions. Finally, Cells were analyzed within 15 minutes by flow cytometer FACS Calibur (BD Biosciences).

Cell cycle analysis

The effect of cytarabine and romidepsin on cell cycle arrest was determined by propidium iodide (PI) staining. Briefly, Molm13 and Molm13-R5 cells were treated with indicated concentrations of cytarabine and romidepsin. Cells were then collected, washed and fixed with 70 % ethanol over night at 4°C. Cells were again washed, re-suspended and incubated with PBS containing RNase (100 ug/ml) followed by incubation with PI (50ug/ml) in dark. Finally, Cells were analyzed immediately by flow cytometer FACS Calibur (BD Biosciences) for cell cycle analysis.

Western blot analysis

Molm13 and Molm13-R5 were seeded at density of 1×10^6 and cultured for 24 h. Cells were treated with different concentrations of reagent understudy either alone or in combination for 24 h. After treatment, cells were collected, washed and lysed on ice with RIPA (radio immunoprecipitation assay) cell lysis reagent supplemented with 1 % phenylmethylsulfonyl fluoride (PMSF). Supernatants were collected in ice chilled tube and protein concentrations were determined using enhanced BCA protein Assay kit (Beyotime, Nanjing, China) by spectrophotometer (Synergy neo multimode microplate reader, BioTek). 25 microgram of proteins were resolved on 10-12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes. Membrane was blocked with 5 % (w/v) nonfat milk for 1 h. Membranes were then incubated overnight at 4°C with cleaved-caspase-3 (1:1,000), PARP (1:1,000), CD123 (1:500) and GAPDH (1:1,000). After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti rabbit second antibody for one hour at room temperature. After washing with TBST, signals were detected using ECL plus chemiluminescence kit by ChemiDoc Imaging System.

Statistical Analysis

The results are expressed as Mean ± SD form five different independent experiments and are statistically compared with untreated control group or compared within treated groups using one-way "ANOVA" followed by "Tukey's Multiple Comparison Test". Columns not sharing the same superscript letters are statistically significant at $P < 0.05$.

Results

QDot labeling map distribution pattern of CD123 on single cell

CD123 protein molecules were labeled by antibody-quantum dot conjugate approach to capture high resolution images of individual CD123 proteins on the surface of Molm13 and Molm13-R5 cells. Figure 1A shows schematic diagram of CD123 labeling by Qdots. Cells were incubated with primary antibody followed by incubation with QDot-conjugated secondary antibody. Flow cytometry results showed that sub-population of CD123+ cells was increased in cytarabine-enriched AML cells (Figure 1B and 1C). Western blot results indicated that the enriched Molm13-R5 cells showed increased expression level of CD123 (Figure 1D). For quantitative profiling of CD123 on single cells, Images were captured under laser scanning confocal microscope (LSCM) and a SIM wide field (SIM-WF) and SIM super resolution imaging model. Figure 1E and 1F show single slice (125 nm in thickness) image of whole cell with QDot-labeled-CD123 molecules under LSCM, SIM-WF and SIM. The data depicted that LSCM gave continuous scattering pattern of CD123 on single cells; however, the resolution was insufficient to extract detailed information of dispersion of CD123 molecules on the surface of single cells. SIM-WF provided further insight where single protein molecules were discriminated. Whereas, SIM super resolution imaging model yielded high resolution images giving detailed information of dispersion pattern of CD123+ molecules as individual dots on the surface of single cells (Figure 1E and 1F). Interestingly, the density pattern of CD123 molecule was increased in Molm13-R5 cells.

SIM imaging and imaris software based analysis reveals different densities of CD123 on Molm13 and Molm13-R5

To determine the distribution pattern and density of CD123 cell surface markers on Molm13 and Molm13-R5 cells, CD123 proteins were labeled with Qdots followed by SIM imaging to imprisonment the optical signals emitted by Qdots. These optical signals provided the location information of CD123 proteins on the surface of single cells which were further analyzed by software imaris to do 3D structure reconstruction and digital simulation. 3D structure reconstruction and digital simulation of Molm13 and Molm13-R5 provided distribution pattern and density of CD123 protein. QDot signals were dispersed randomly throughout the cell membrane (Figure 2A and 2B). The density of CD123 protein on Molm13 and Molm13-R5

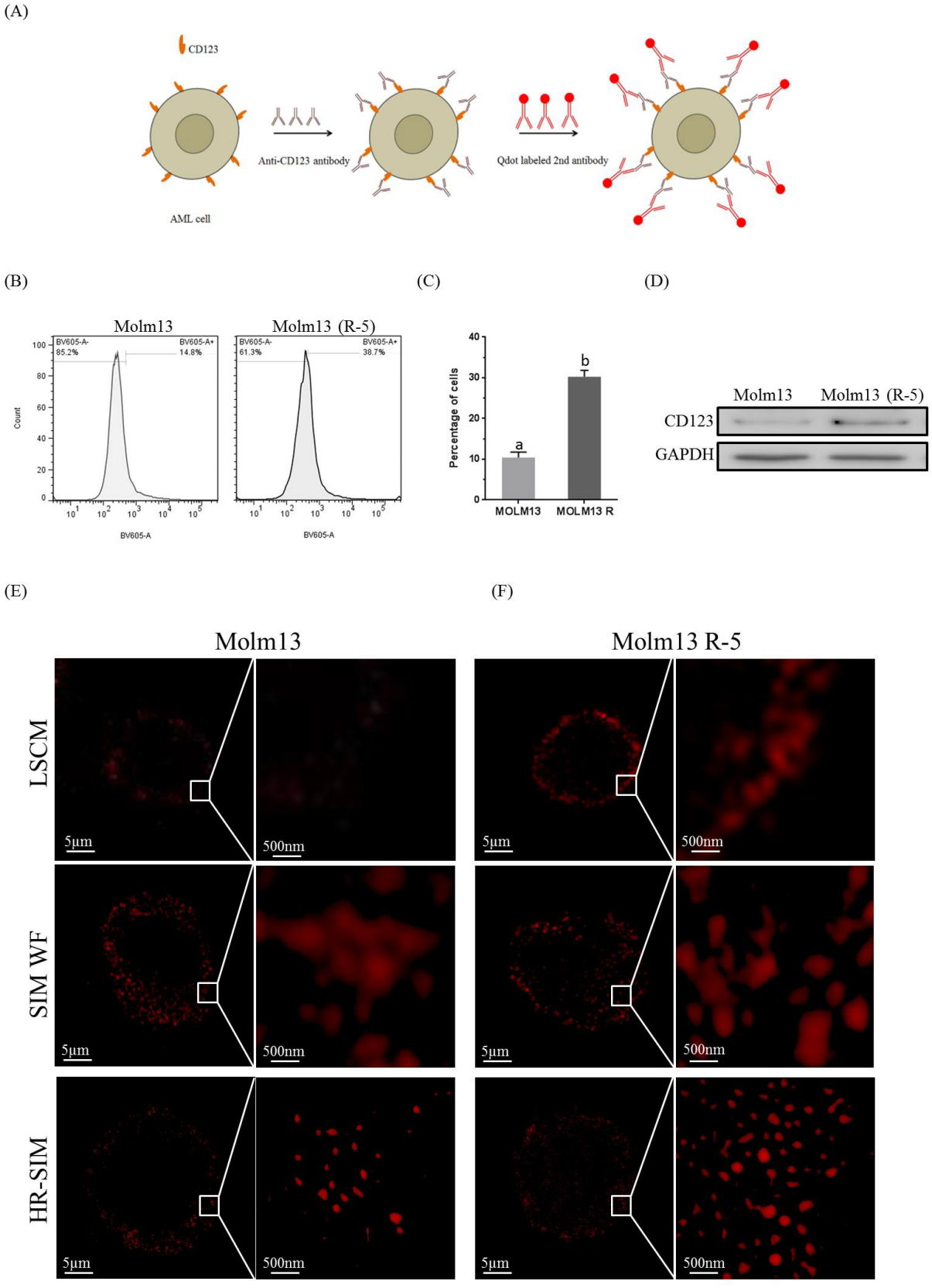

Figure 1. Schematic Representation of QDot Staining and CD123 Images on Single Cell. (A) Schematic diagram of CD123 labeling. (B,C) Flow cytometry analysis and percentage of subpopulation of CD123+ cells in Molm13 and Molm13-R5 enriched cells. Column sharing not same superscript letter differ significantly ($P < 0.05$). (D) Western bolt analyses of CD123 expression in Molm13 and Molm13-R5. (E,F) Slice section and zoomed in images under the laser scanning confocal microscope, SIM wide field and SIM of Molm13 and Molm13-R5 single cell, respectively.

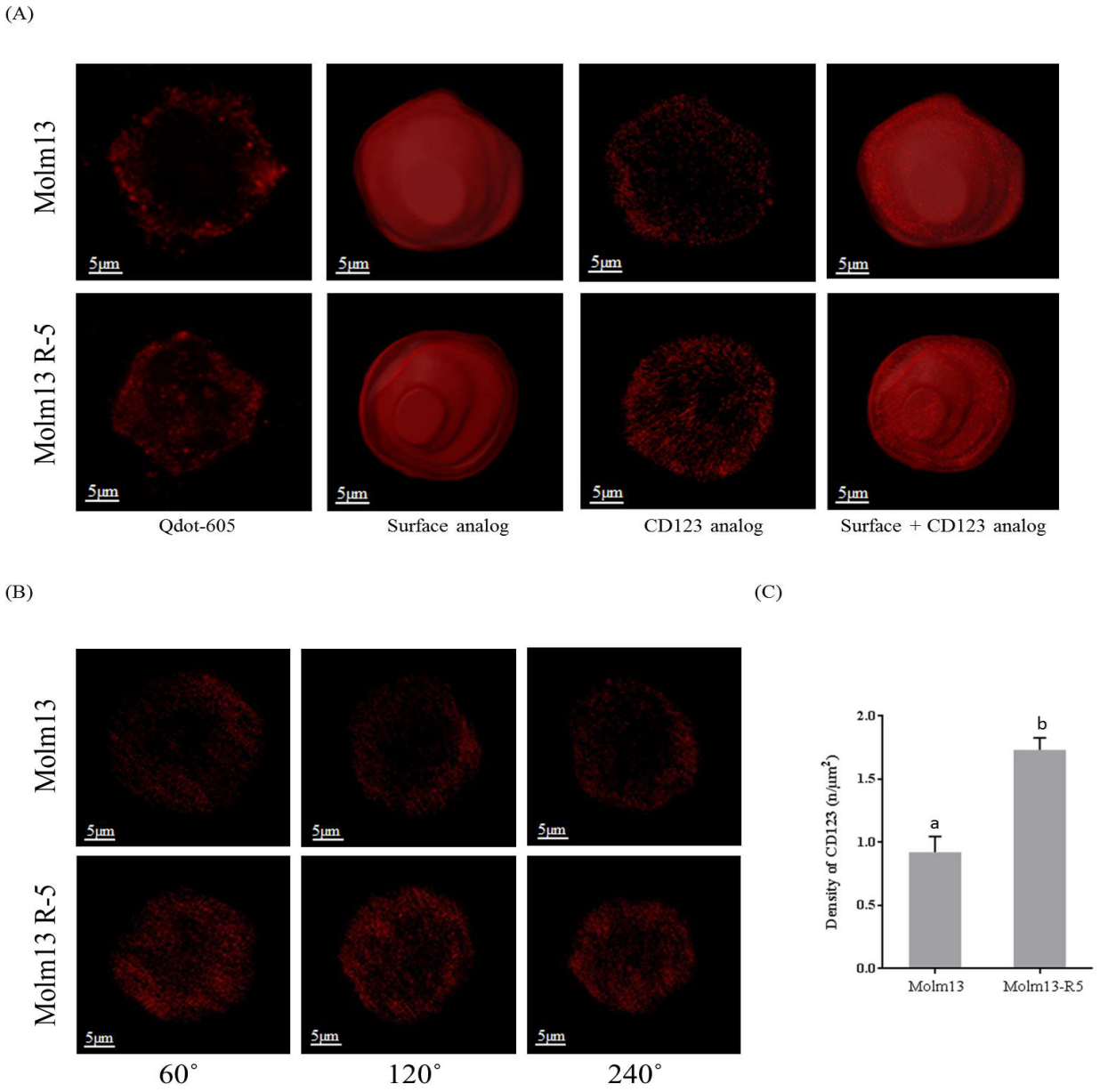

Figure 2. Quantitative Profiling and 3D Structure Reconstruction of CD123 on Molm13 and Molm13 R-5 Enriched Cells by SIM and Software Imaris. (A) Dispersion pattern of CD123 on the surface of single cell reconstructed from the raw SIM image. (B) Density and dispersion pattern of CD123 on Molm13 and Molm13-R5 single cells at angles of 60°, 120° and 240°. (C) Comparison of CD123 density on the surface of single cells for Molm13 and Molm13-R5 cells. Column sharing not same superscript letter differ significantly ($P < 0.05$).

cell is 0.92 and 1.73 per μm^2 respectively (Figure 2C).

Cytarabine and romidepsin inhibits proliferation of AML cells

The growth inhibitory effect of cytarabine and romidepsin against Molm13 and Molm13-R5 cells was evaluated by MTT assay. The data (Figure 3A) showed that cytarabine inhibited the proliferation of Molm13 cells in dose-dependent manner with IC_{50} value 400 nM. Figure 3B showed that romidepsin inhibited the growth of Molm13 and Molm13-R5 cells in dose-dependent manner with IC_{50} value 5 and 8 nM respectively.

Romidepsin augments the cytotoxicity of cytarabine in Molm13 and Molm13-R5 cells

During enrichment, the proportion of cells expressing CD123 cell surface protein was increased in Molm13-R5 cells. There is growing evidence that romidepsin targets both CD123− and CD123+ cells (Yan et al., 2019), therefore, we wondered if romidepsin could sensitized Molm13-R5 cells to cytarabine. The data showed that romidepsin sensitized Molm13 and Molm13-R5 cells to cytarabine and augmented the effect of cytarabine (Figure 3C and 3D). The result was further assessed by evaluating apoptosis effect of romidepsin and cytarabine on Molm13 and Molm13-R5 cells by flow cytometry. Cells were treated with indicated concentrations of romidepsin and cytarabine followed by Annexin V-FITC and PI staining

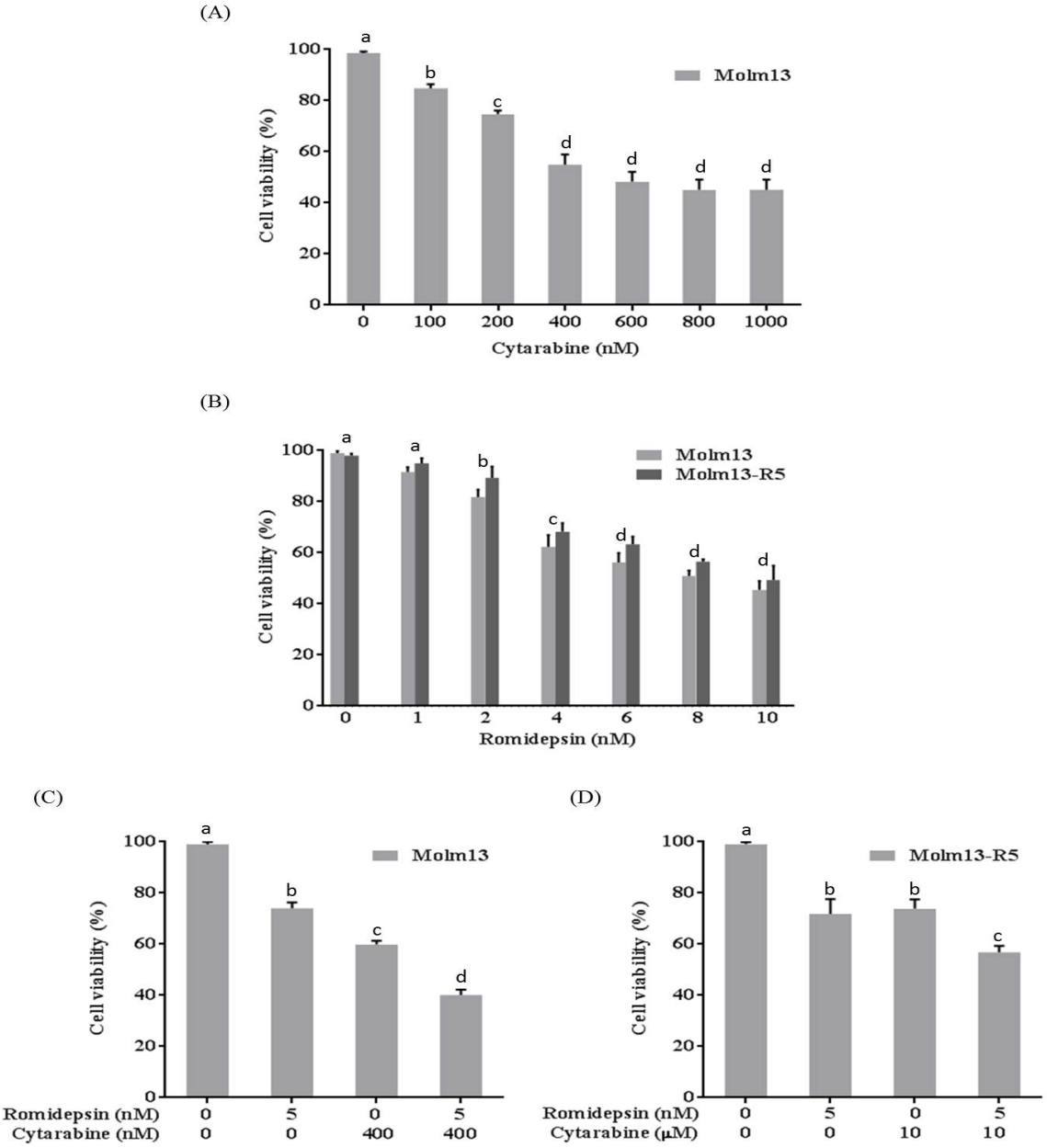

Figure 3. Growth Inhibitory Effect of Drugs on AML Cells (A) Growth inhibitory effect of cytarabine on Molm13 cells at 48 h. (B) Growth inhibitory effect of romidepsin on Molm13 and Molm13-R5 enriched cells at 48 h. (C,D) Synergistic effect of cytarabine and romidepsin on Molm13 and Molm13-R5 cells at 48. Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P < 0.05$).

for flow cytometry analysis. The data depicted that (Figure 4A and 4B) romidepsin augmented the apoptosis inducing activity of cytarabine with concomitant increased expression of cleaved-caspase-3 and PAPR cleavage in Molm13 cells (Figure 4C). Furthermore, romidepsin sensitized Molm13-R5 cells to cytarabine and induced strong apoptosis activity with concomitant increased expression of cleaved-caspase-3 and PAPR cleavage (Figure 4D, 4E and 4F).

Romidepsin and cytarabine causes G0/G1 cell phase arrest

Cell cycle analysis was performed to know the effect of romidepsin and cytarabine either alone or in combination on Molm13 and Molm13-R5 cells by flow cytometry. Untreated groups of Molm13 and Molm13-R5 cells were compared which showed that cytarabine-resistant Molm13 cells were growing in the manner as that of Molm13 cells (data not shown). The data (Figure 5A and 5B) showed that Romidepsin and cytarabine arrested Molm13 cells at G0/G1 phase. The population of cells in G0/G1 phase was significantly increased on combine treatment of romidepsin and cytarabine. Cell cycle analysis was also performed for Molm13-R5 cells. The data indicated that romidepsin significantly increased the population of cells at G0/G1 phase than that of cytarabine, while combine treatment further increased population of cells at G0/G1 phase (Figure 5C and 5D).

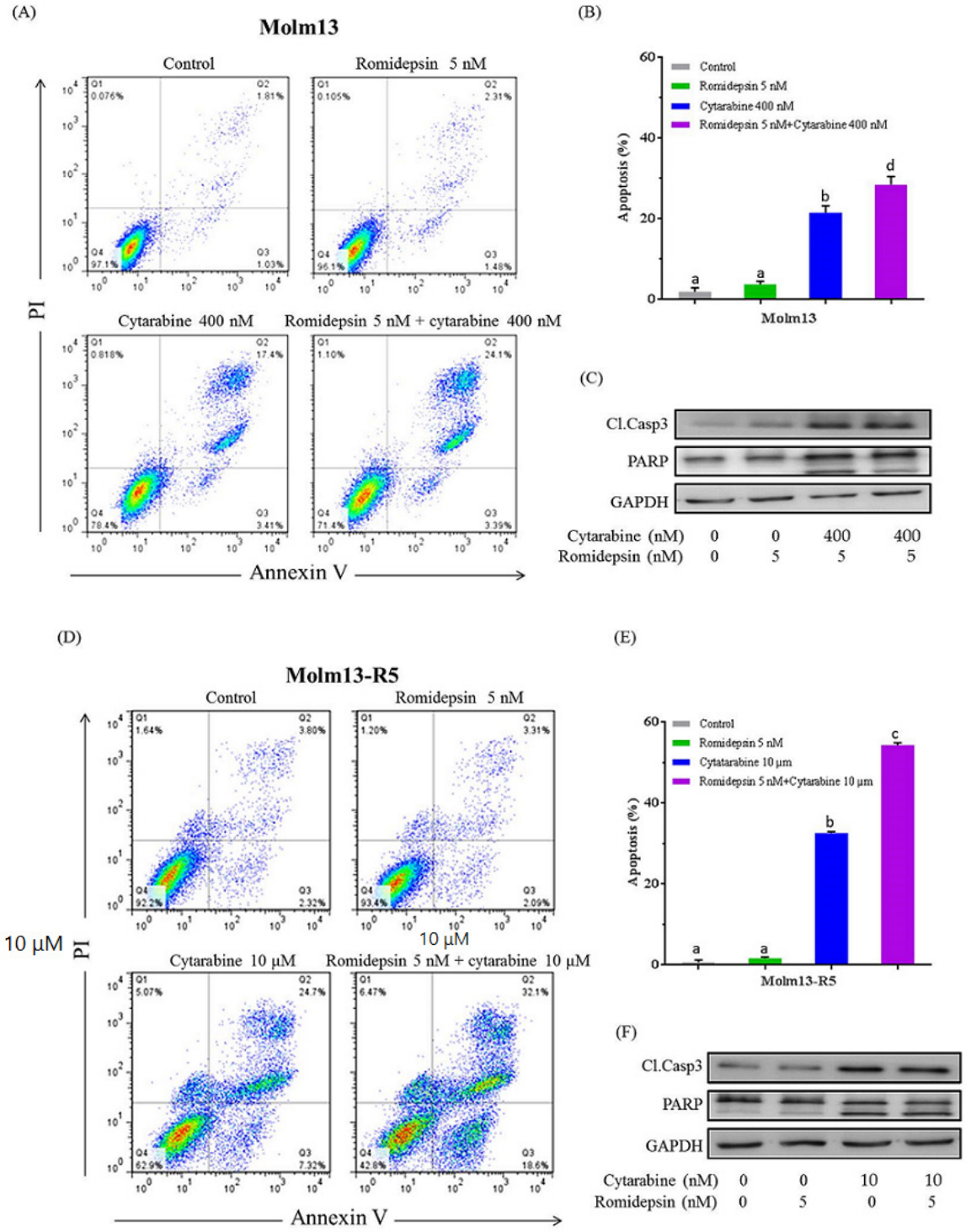

Figure 4. Flow Cytometry Analysis of Apoptosis Induced by Drugs at 48 h. (A) Apoptosis induced by cytarabine and romidepsin in Molm13 cells. (B) Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P < 0.05$). (C) Western blot analyses of cleaved caspase-3 and PARP in Molm13 cells. (D) Apoptosis induced by cytarabine and romidepsin in Molm13-R5 enriched cells. (D) Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P < 0.05$). Western blot analyses of cleaved caspase-3 and PARP in Molm13-R5 cells.

Discussion

Quantitative profiling of cell surface markers is gaining importance in characterization of tumor cells heterogeneity and single cell biology. Differential expression of cell surface markers eliminates the challenge of tumor cell targeted therapies (Cohen et al., 2017). Several single molecule imaging techniques including STED, PALM and STORM can provide the detailed information of protein localization on the cell surface but the requirement of photo-stable fluorescent probes and high intensity illumination may influence the actual

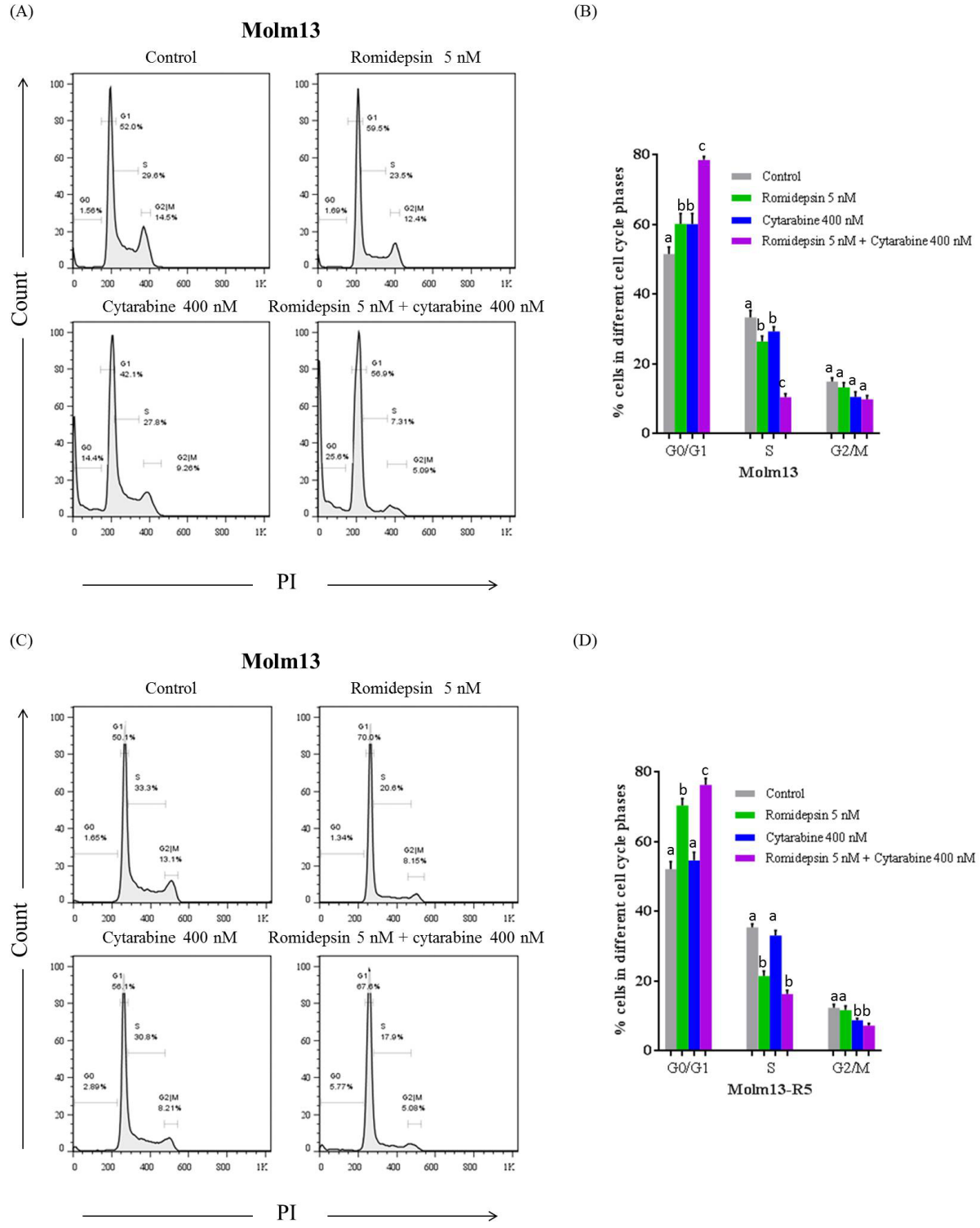

Figure 5.Flow Cytometry Analyses of Cell Cycle Induced by Drugs at 48 h. (A) Cell cycle changes induced by cytarabine and romidepsin in Molm13 cells. (B) Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P < 0.05$). (C) Cell cycle changes induced by cytarabine and romidepsin in Molm13-R5 enriched cells. (D) Data are expressed as mean ± SD of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P < 0.05$).

information of the specimen (Igarashi and Nozumi, 2018). Here, we used newly developed technique of labeling the cell surface proteins by Qdots followed by high resolution SIM imaging to get detailed information of localization of single molecule. QDots as semiconductor luminescent material with narrow and bright spectra provides the solution to overcome the problem of photo-stable fluorescent probes. SIM reconstructs the fine structure of cells by computing the interference conformations induced by irradiation with striped-pattern excitation light

and provides quantitative information of single molecule. Using this new approach we determined the density of CD123 protein molecules on the surface of Molm13 and Molm13-R5 cells.

Treatment of patients with acute myeloid leukemia (AML) has changed little over the past 4 decades. Induction chemotherapy and best supportive care has resulted in complete remission and improved the survival of AML patients. Despite complete remission, relapse of AML occurs (Ma et al., 2019). Cytarabine is the first line chemotherapeutic drug that has been used in induction chemotherapy for AML since 1960s (Li et al., 2017; Yan et al., 2019). It has been shown that higher level of CD123 is strongly correlated with chemo-resistance and lower survival rate and the proportion of CD123+ sub-population at diagnosis determines the rate of complete remission and relapse (Li et al., 2017; Yan et al., 2019). Hence, we enriched a drug-resistant cell line from a drug-sensitive AML cell line Molm13, by exposing the cell with gradually increasing the concentration of cytarabine. The morphology of cells was not changed (data not shown) after several generations. Combine data form flow cytometry, QDot labeling followed by SIM super resolution imaging and Imaris digital simulation suggested that sub-population of Molm13 cells with CD123+ phenotype were enriched and showed acquired resistance against cytarabine.

We found that proportion of sub-population of cells with higher densities of CD123 on cells surfaces was increased in cytarabine-enriched Molm13 cells as compared to parent Molm13 cells. The differential expression of CD123 in sub-populations of AML cells changes the phenotype of cells. Growing evidences suggest that CD123 expression in sub-populations of AML cells cause them to develop more resistance against chemotherapeutic drugs. It is well established that romidepsin targets both CD123− and CD123+ sub-populations of AML cells (Yan et al., 2019). Together with this notion, our results depict that romidepsin produced the same effect and also sensitized and augmented the apoptosis activity of cytarabine in Molm13 and Molm13-R5 cells. Targeting both CD123− and CD123+ populations might reduce the risk of relapse of leukemia that need to be explored further at preclinical and clinical level.

In conclusion, quantitative profiling of cell surface protein molecules by QDot labeling and SIM super resolution imaging is trustworthy approach for in situ characterization of tumor cells heterogeneity. Besides, we determined that the enriched Molm13 cells showed higher density of CD123 on cells surfaces as compared to parental Molm13 cell populations. Further molecular level studies are needed to know the mechanism of CD123-associated chemoresistance against cytarabine in sub-populations of AML cells. More leukemia cells markers should be labeled and quantify to get more understanding of their localization on the cell surface and involvement in drug resistance which may help in targeted chemotherapy.

Author Contribution Statement

TM, and XE.Z conceptualized the study. TM executed the experiments. TM performed data analysis and drafted the manuscript. XE.Z revised the manuscript. All authors reviewed and approved the final manuscript for submission.

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Data Availability

All relevant data are in the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of research article.

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