# Raised CD40L expression attenuates drug resistance in Adriamycin-resistant THP-1 cells

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**Abstract.** Acute myeloid leukemia is a common hematological malignancy that often exhibits strong drug resistance when treated using conventional chemotherapy. Although numerous studies have been carried out to develop methods of overcoming drug resistance, the results have generally been unsatisfactory. CD40 ligand (CD40L) has been shown to improve the sensitivity of cancer cells to drug treatment. In the present study, Adriamycin (ADM)-resistant human monocytic THP-1 cells (THP-1/A cells) were developed by incubating THP-1 cells with increasing concentrations of ADM. Cells were transfected with CD40L vectors to explore the potential involvement of CD40L in regulating multidrug resistance (MDR) in cancer. Cell proliferation and viability were measured using the Cell Counting Kit-8 assay; cell apoptosis was evaluated by flow cytometry, trypan blue staining and caspase-3 activity; and the expression of MDR-associated protein 1 (MRP1) and permeability glycoprotein (P-gp) was analyzed using western blotting. The results revealed that the protein expression levels of MRP1 and P-gp were downregulated by raised CD40L expression and that the combination of raised CD40L expression with daunorubicin (DNR), a drug from which ADM is derived, significantly increased the extent of cell apoptosis, indicating that drug resistance was effectively attenuated by CD40L. Collectively, these results suggested that CD40L may contribute towards reducing DNR resistance in THP-1/A cells.

## Introduction

Acute myeloid leukemia (AML) is a common hematological malignancy characterized by an increase in the number of undifferentiated cells in the bone marrow and peripheral blood (1). AML-M5, an AML subtype, has a mortality rate of nearly 90% (1-3). One of the main clinical techniques used to treat AML is conventional chemotherapy. Although allogeneic hemopoietic stem cell transplantation has emerged as an alternative (4), it is costly, and finding suitable donors for transplantation is difficult. Both treatment methods face the challenge of strong drug resistance (5). While many researchers have attempted to address this issue, there is still a lack of effective drugs for the treatment of AML; therefore, it is necessary to develop strategies to overcome drug resistance in AML therapy.

CD40 ligand (CD40L), otherwise known as CD154, is a 33-kDa type II transmembrane protein expressed on the membranes of T lymphocytes, and belongs to the tumor necrosis factor superfamily (6). CD40 is the CD40L receptor, and is expressed on the membranes of B lymphocytes and macrophages (7). CD40 has been observed not only in normal healthy cells, but also in cancer cells, such as those from melanoma, prostate cancer, lung cancer, bladder cancer, cervical cancer, ovarian cancer, lymphocytic leukemia, lymphoma, multiple myeloma and AML (7). The CD40/CD40L axis plays important roles in immunity and inflammation, and exhibits both pro- and anti-neoplastic activity in many diseases and cancer types (8). It has also been reported that CD40L improves the sensitivity to drugs used in the treatment of stomach cancer (9); however, to the best of our knowledge no studies have been conducted into the role of CD40L in AML-M5 treatment.

In this study, the involvement of CD40L in the drug resistance mechanisms of AML-M5 cells was explored. THP-1 cells were rendered resistant to Adriamycin (ADM), a common chemotherapeutic agent, and treated with daunorubicin (DNR), a drug with a highly similar structure and the same pharmacological effects as ADM. The effects of CD40L on drug treatment and DNR resistance were evaluated by inducing raised CD40L expression in THP-1 cells, and assessing apoptosis and the expression of drug resistance-related genes.

## Materials and methods

*Preparation of ADM-resistant THP-1/A cells.* The human monocytic THP-1 cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in an atmosphere containing

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5% $CO_2$ and 95% air at 37˚C. To obtain ADM-resistant cells (denoted THP-1/A cells), normal THP-1 cells were treated with an ADM concentration gradient (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 $\mu$g/ml) as previously described (10-13). When the ADM concentration was 1.2 $\mu$g/ml, most of the cells grew stably. When the ADM concentration was further increased, THP-1 cell apoptosis increased significantly and cell morphology changed. Therefore, 1.2 $\mu$g/ml was chosen as the intervention concentration (preliminary study; data not shown). The THP-1/A cells were then cultured in an ADM-free medium for 2 weeks before being used in the subsequent experiments.

*Vector construction and transfection*. The CD40L sequence (accession no. NM_000074.2, CD40L sequence: 5'-ATG ATCGAAACATACAACCAAACTTCTCCCCGATCTGCG GCCACTGGACTGCCCATCAGCATGAAAATTTTTATG TATTTACTTACTGTTTTTCTTATCACCCAGATGATT GGGTCAGCACTTTTTGCTGTGTATCTTCATAGAAGG TTGGACAAGATAGAAGATGAAAGGAATCTTCATGAA GATTTTGTATTCATGAAAACGATACAGAGATGCAA CACAGGAGAAAGATCCTTATCCTTACTGAACTGTGA GGAGATTAAAAGCCAGTTTGAAGGCTTTGTGAAGGA TATAATGTTAAACAAAGAGGAGACGAAGAAAGA AAACAGCTTTGAAATGCAAAAAGGTGATCAGAATCC TCAAATTGCGGCACATGTCATAAGTGAGGCCAGCAG TAAAACAACATCTGTGTTACAGTGGGCTGAAAAAGG ATACTACACCATGAGCAACAACTTGGTAACCCTGGA AAATGGGAAACAGCTGACCGTTAAAAGACAAGG ACTCTATTATATCTATGCCCAAGTCACCTTCTGTTC CAATCGGGAAGCTTCGAGTCAAGCTCCATTTATAGC CAGCCTCTGCCTAAAGTCCCCCGGTAGATTCGAGAG AATCTTACTCAGAGCTGCAAATACCCACAGTTCCG CCAAACCTTGCGGGCAACAATCCATTCACTTGGGAG GAGTATTTGAATTGCAACCAGGTGCTTCGGTGTTTG TCAATGTGACTGATCCAAGCCAAGTGAGCCATGGCA CTGGCTTCACGTCCTTTGGCTTACTCAAACTCTGA-3') was obtained from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore). The human CD40L gene cDNA was inserted into the pHBLV-CMVIE-Zs Green-T2A-Puro lentiviral transfer vector (Hanbio Biotechnology Co., Ltd.), which emits green fluorescence at the restriction sites for *Eco*RI and *Bam*HI. Vector stocks were produced via the calcium-phosphate transient transfection of 293T cells (2-5x$10^4$ cells) at 70-80% confluence. Cells were transfected with vectors at 50 TU/ml by using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Stably transduced THP-1/A cells were maintained under G418 selection (200 $\mu$g/ml) for 12 days at 37˚C, 5% $CO_2$ incubator, and the transfection rate was observed by western blotting and fluorescence microscopy (DMIL LED; Leica Microsystems GmBH).

*Cell Counting Kit-8 (CCK-8) assay*. To confirm the successful preparation of ADM-resistant THP-1/A cells, the degree to which THP-1 and THP-1/A cell proliferation was inhibited after ADM treatment was evaluated using the CCK-8 assay (Beyotime Institute of Biotechnology). ADM was administered to THP-1 and THP-1/A cells at concentrations of 0.5, 2, 4, 8 and 16 $\mu$g/ml for 24 h at 37˚C. Subsequently, 10 $\mu$l CCK-8 solution was added and the optical density was measured at a wavelength of 450 nm. Inhibition of cell proliferation (%) was calculated using the following formula: $[1-(OD_{experimental}-OD_{blank}/OD_{control}-OD_{blank})]$ x100%.

The effect of CD40L on THP-1/A cells was explored by transfecting with negative control (NC) lentiviral vectors (LV) or CD40L-LV. The CCK-8 assay was performed to determine the viability of THP-1/A, NC-LV THP-1/A and CD40L-LV THP-1/A cells between days 0 and 7. Additionally, whether CD40L affected the resistance of THP-1/A cells to chemotherapeutic agents was examined, using DNR as a model drug. Cells were cultured in 24-well plates at a density of 1x$10^5$ cells/ml, and treated with DNR at 0, 0.5, 2, 4, 8, 16, 32 or 64 $\mu$g/ml for 24 h at 37˚C. The proliferation of the DNR-treated THP-1/A, NC-LV THP-1/A and CD40L-LV THP-1/A cells was then determined using the CCK-8 assay, with results presented as the degree of cell proliferation inhibition (%).

*Cell apoptosis*. To investigate the effect of DNR on THP-1/A cells transfected with CD40L vectors, apoptosis was measured using an Annexin V/propidium iodide (PI) kit (Beyotime Institute of Biotechnology). Cells were divided into six groups: THP-1/A, NC-LV THP-1/A, CD40L-LV THP-1/A, THP-1/A + DNR, NC-LV THP-1/A + DNR and CD40L-LV THP-1/A + DNR. Cells (1x$10^5$ cells/ml) were seeded into 24-well plates and treated with normal saline or 4 $\mu$g/ml DNR once they had reached 70% confluence. After 24 h, 5 $\mu$l Annexin V and 5 $\mu$l PI were added to each well, the cells were incubated for 20 min in the dark at 4˚C and apoptosis was determined by flow cytometry (FC500 MCL flow cytometer with, CXP Analysis Software Version 2.2; Beckman Coulter, Inc.).

*Western blotting*. Total protein was extracted using a total protein extraction kit (ProteoPrep; Sigma-Aldrich; Merck KGaA), and the protein concentration was measured using a 3,3'-diaminobenzidine tetrahydrochloride kit (Beyotime Institute of Biotechnology). Proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk at 4˚C for 2 h, and incubated with primary and secondary antibodies for 2 h each at room temperature. The following primary and secondary antibodies were used: Anti-CD40L (Abcam; ab65854; 1:500), anti-multidrug resistance (MDR)-associated protein (MRP) 1 (Abcam; ab84320; 1:1,000), anti-permeability glycoprotein (P-gp; Abcam; ab129450; 1:5,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (Abcam; ab6721; 1:20,000). Images of protein bands were captured using an ECL color detection kit (cat. no. WBKLS0010; EMD Millipore) and the Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology Co., Ltd.).

*Caspase-3 activity*. The caspase-3 activity of the six aforementioned experimental groups was determined using a caspase-3 colorimetric assay kit (Nanjing Keygen Biotech Co., Ltd.), according to the manufacturer's instructions.

*Trypan blue staining*. Trypan blue dye (Beyotime Institute of Biotechnology) was used to evaluate cell death in the six aforementioned experimental groups. A total of $10^6$ cells/well were pretreated with normal saline or 4 $\mu$g/ml DNR at room

temperature for 24 h, in 24-well plates, harvested, mixed with 0.4 % trypan blue, and counted using a hemocytometer under an optical microscope (x200).

*Statistical analysis*. All data are from three replicates and presented as the mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Duncan's multiple comparisons test using SPSS 19.0 (IBM Corp.). All figures were prepared using GraphPad Prism 5.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Vector transfection efficiency*. To verify the transfection efficiency of the CD40L expression vector, the green fluorescence from the lentiviral vectors was visualized under a fluorescence microscope (Fig. 1A). Green fluorescence was observed in both the NC-LV and CD40L-LV THP-1/A cells, indicating that both of the fluorescent lentiviral vectors had successfully transfected a number of the THP-1A cells. Fig. 1B shows that CD40L protein expression in the CD40L-LV THP-1/A cells appeared higher than that in the non-transfected cells or those transfected with NC vectors, suggesting that raised CD40L expression was achieved via transfection.

*CD40L suppresses THP-1/A cell proliferation*. The CCK-8 assay (Fig. 2A) revealed that cell proliferation was inhibited to a significantly lower level in THP-1/A cells than that in THP-1 cells (P<0.05) when treated with various ADM concentrations. This result suggested that when THP-1 cells were rendered resistant to ADM (THP-1/A cells), the effect of the ADM treatment was attenuated due to resistance having been developed. This, in turn, led to greater proliferation of the cells. CD40L vectors were transfected into the ADM-resistant cells, and it was observed that the viability of the CD40L-LV THP-1/A cells was lower than that of the untransfected and NC-LV-transfected THP-1/A cells over a 7 day period (P<0.05; Fig. 2B). There was no difference between the viability of THP-1/A and NC-LV THP-1/A cells during this period.

*CD40L enhances the inhibitory effects of DNR on THP-1/A cells*. To explore whether CD40L contributes to the inhibitory effect of DNR on THP-1/A cell growth, the proliferation of THP-1/A, NC-LV THP-1/A and CD40L-THP-1/A cells was measured after treatment with various concentrations of DNR. The results shown in Fig. 2C suggest that, upon DNR treatment, the proliferation of THP-1/A cells transfected with CD40L vectors was inhibited to a significantly greater extent than that of both the non-transfected or NC-LV-transfected cells (P<0.05). These results indicated that DNR exerted a stronger effect when CD40L expression was raised, suggesting that the drug-resistant properties of THP-1/A cells were suppressed by CD40L.

*Raised CD40L expression induces THP-1/A cell apoptosis*. The apoptosis of THP-1/A cells with or without transfection of CD40L vectors and DNR treatment was assessed by flow cytometry, caspase-3 activity colorimetric assays and trypan blue staining. In this study, CD40L transfection increased the apoptosis levels in cells that had not been treated with DNR. The flow cytometry results demonstrated that THP-1/A cells had a certain degree of resistance to DNR, but transfection with CD40L vectors (CD40L-LV THP-1/A + DNR) significantly promoted apoptosis (P<0.05), indicating that DNR resistance was attenuated by CD40L (Fig. 3A and B). Similar results were obtained by trypan blue staining (Fig. 3C), in which positive staining represents dead cells. The percentage of trypan blue-positive cells transfected with CD40L-LV and treated with DNR was higher than for DNR-treated cells that were untransfected or were transfected with NC-LV (P<0.05). Furthermore, the colorimetry assay revealed that the activity of caspase-3, a prominent pro-apoptotic protein, was enhanced in DNR-treated THP-1 cells transfected with the CD40L vector (Fig. 3D; P<0.05). These results collectively suggested that CD40L may suppress DNR resistance in THP-1/A cells.

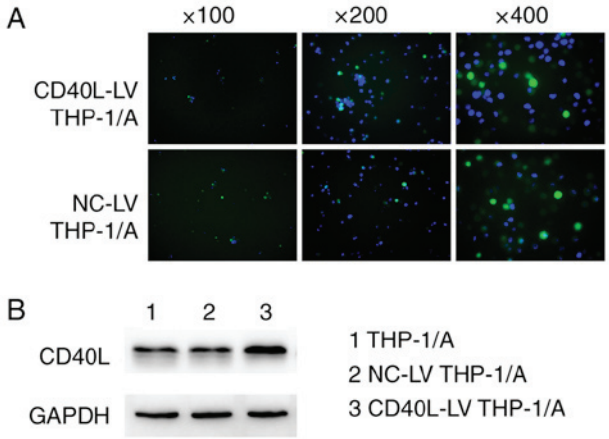


Figure 1. CD40L expression was raised in THP-1/A cells. (A) Green lentiviral vector fluorescence was observed in NC-LV and CD40L-LV-transduced THP-1/A cells. (B) The protein expression of CD40L in CD40L-LV-transfected THP-1/A cells appeared increased compared to untreated THP-1/A and NC-LV-transfected THP-1/A cells. CD40L, CD40 ligand; LV, lentivirus; NC, negative control.

*CD40L downregulates the expression of drug resistance-related genes*. To further explore drug resistance in THP-1/A cells, the protein expression levels of MRP1 and P-pg were measured (Fig. 4), two proteins which are associated with MDR. Fig. 4 shows that the protein expression levels of MRP1 and P-pg appear attenuated in THP-1/A cells after CD40L-LV transfection. The reduction appeared even more prominent following treatment with DNR, indicating that raised CD40L expression effectively downregulated the expression levels of MRP and P-gp.

## Discussion

After long-term exposure to chemotherapeutic drugs, tumors can become resistant and lose sensitivity to other antineoplastic drugs with different structures and mechanisms in a process known as MDR. Although progress has been made in AML therapy, appropriate and effective treatment methods for the disease are lacking, with the issue of drug resistance being a major concern. MDR is a major obstacle to the treatment of leukemia and the main cause of its recurrence (14). It has been previously found that the drug resistance index of leukemia cells

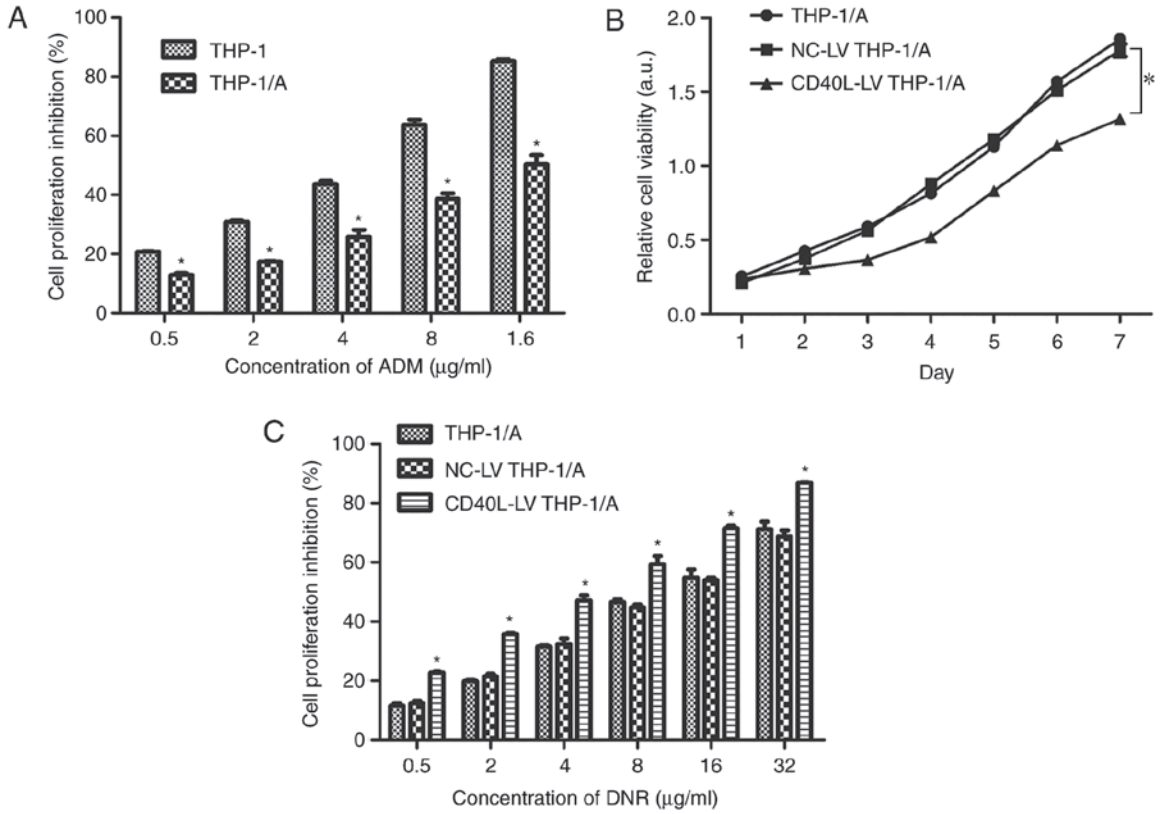


Figure 2. Proliferation of THP-1 cells subjected to ADM resistance, CD40L vector transfection and DNR treatment. (A) The extent of cell proliferation inhibition (%) in THP-1 and THP-1/A cells was detected using a Cell Counting Kit-8 assay (n=3). *P<0.05 vs. THP-1. (B) The viability of THP-1/A, NC-LV THP-1/A, and CD40L-LV THP-1/A cells was evaluated once per day for 7 days. (C) The extent of cell proliferation inhibition (%) in THP-1/A, NC-LV THP-1/A and CD40L-LV THP-1/A cells, treated with DNR (0-32 μg/ml), was evaluated using a Cell Counting Kit-8 assay. *P<0.05 vs. NC-LV THP-1/A cells (n=3). ADM, Adriamycin; CD40L, CD40 ligand; DNR, daunorubicin; LV, lentivirus; NC, negative control.

to doxorubicin was as high as 182 (15). Styczyński *et al* (16) tested the anti-leukemia activity of five glucocorticoids in 25 AML cell samples, and found that there was significant cross-resistance against all of the glucocorticoids in all samples. The ATP-binding cassette transmembrane protein family is the largest family of transmembrane proteins (17) and includes P-gp, MDR-related proteins and lung resistance proteins. Raised expression of ATP-binding cassette transmembrane proteins is recognized as the main cause of MDR in leukemia cells (18). P-gp can be detected in almost all drug-resistant leukemia strains and has a very broad range of substrates, including ADM, epirubicin, docetaxel and other anti-cancer drugs (19). Leukemia cell MDR is also closely related to the activity of the apoptotic gene p53 and NF-κB.

Both CD40L and soluble CD40L generated from surface-expressed CD40L interact with the CD40 receptor, resulting in the activation of various pro-inflammatory responses (20). Qin *et al* (21) reported that soluble CD40L enhanced the drug sensitivity of ovarian cancer cells, whilst during bladder cancer treatment in a mouse model, simultaneous CD40L and 5-fluorouracil treatment resulted in an enhanced therapeutic effect compared to 5-fluorouracil treatment alone (22). However, few studies have examined the effect of CD40L on MDR in AML-M5.

ADM (23) and DNR (24) are important and commonly-used drugs in the clinical treatment of AML. In this study, THP-1 cells were rendered resistant to ADM (THP-1/A cells) and served as a cellular model of drug-resistant AML-M5 (3,25). The cells underwent transfection to raise CD40L expression, and the resulting changes in drug resistance were elucidated via DNR administration. The CCK-8 assay and flow cytometry were used to measure cell growth and apoptosis. It was revealed that CD40L inhibited THP-1/A cell growth and enhanced DNR-induced cell death. Furthermore, since the drug resistance-related proteins MRP1 and P-pg are highly expressed in drug-resistant cells (26,27), the effect of CD40L and DNR on the activity of these proteins in THP-1 cells was investigated. Following DNR treatment, the expression levels of MRP1 and P-pg in THP-1/A cells with raised CD40L expression were lower than those in normal non-transfected cells, suggesting that CD40L successfully suppressed the DNR resistance of THP-1/A cells. Due to the pivotal role of P-gp in MDR, current research on reversing drug resistance in leukemia has mainly focused on inhibiting P-gp expression. Thus far, studies have been carried out on four generations of P-gp inhibitors. The first generation consists of verapamil (a calcium channel blocker) and cyclosporin A (an immunosuppressant); the second generation consists of dextral verapamil and valspodar (28,29); the third generation consists of tariquidar and laniquidar (30); and the fourth generation consists of alkanol compounds (31). Despite this research, little has been achieved due to the considerable side effects and low inhibition ability of these P-gp inhibitors. Reversing MDR by altering drug accumulation in drug-resistant cells is also a focus of current research. Increased

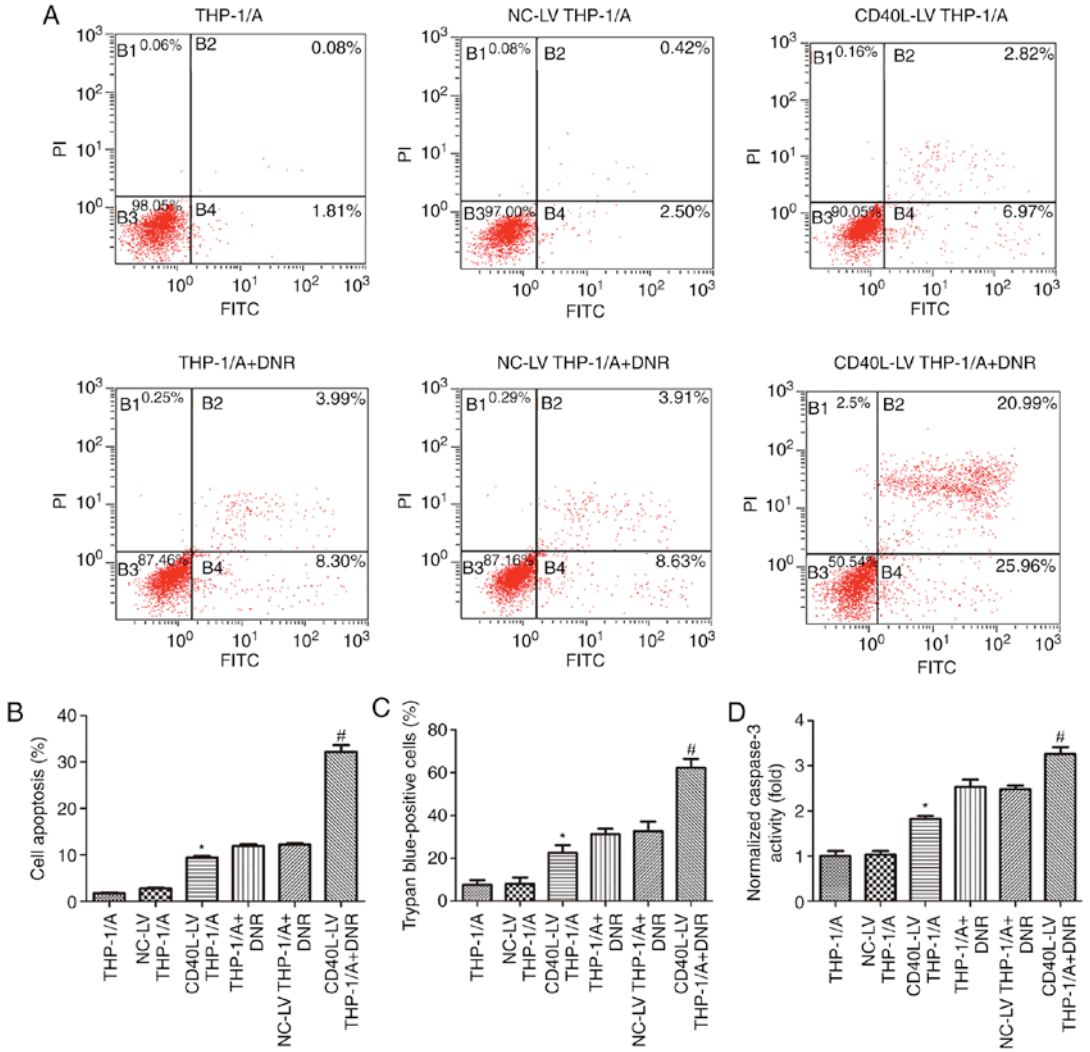


Figure 3. Combined effect of CD40L and DNR on apoptosis and caspase-3 activity in THP-1/A cells. (A) The percentage of apoptotic THP-1/A, NC-LV THP-1/A, and CD40L-LV THP-1/A cells, with or without DNR treatment, was measured by flow cytometry and (B) quantified accordingly (n=3). (C) Apoptosis was further assessed by trypan blue staining. (D) Caspase-3 activity was evaluated using a colorimetric caspase-3 activity kit. *P<0.05 vs. NC-LV THP-1/A cells; #P<0.05 vs. NC-LV THP-1/A+DNR (n=3). CD40L, CD40 ligand; DNR, daunorubicin; LV, lentivirus; NC, normal control; PI, propidium iodide.

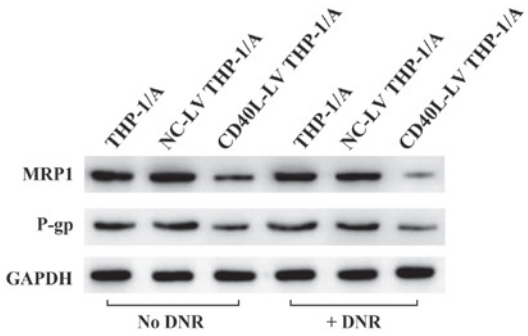


Figure 4. Effect of CD40L on the DNR resistance of THP-1/A cells. The protein expression levels of MRP1 and P-gp were measured by western blot analysis. CD40L, CD40 ligand; DNR, daunorubicin; LV, lentivirus; MRP1, multidrug resistance-associated protein 1; NC, normal control; P-gp, permeability glycoprotein.

expression of ATP-binding cassette-associated proteins can reduce the concentration of therapeutic drugs within cells, thereby resulting in failure to achieve the therapeutic effects and leading to drug resistance (32). Styczynski *et al* (33) found that the concentrations of the drugs idarubicin (IDA) and DNR were lower in drug-resistant AML cells, and that raised P-gp expression decreases the IDA and DNR concentrations. When multiple drugs are used in combination, the sensitivity of leukemia drug-resistant cells to chemotherapeutic drugs can be increased (34). In this study, the protein expression of MRP1 and P-gp in the DNR group (without CD40L) did not change significantly; however, the protein expression of MRP1 and P-pg in the raised CD40L expression group (without DNR) and CD40L + DNR group appeared to decrease, particularly in the CD40L + DNR group. These results suggested that raised CD40L expression not only inhibits MRP1 and P-gp expression, but also promotes the DNR sensitivity of AML drug-resistant cells. Raised CD40L may inhibit the drug-resistance of AML cells by inhibiting P-gp expression. Whether raised CD40L expression can promote the accumulation of DNR in drug-resistant AML cells and alter their drug release system remains to be elucidated.

In the present study, the effect of CD40L on drug resistance in ADM-resistant THP-1 cells was observed, however the use of multiple cell lines was beyond the scope of the

present study. The use of a greater number of cell lines would allow for further verification that raised expression of CD40L attenuates drug resistance in AML-5M. In conclusion, CD40L may enhance the anti-tumor properties of DNR and may be a possible solution for treating drug resistance in AML by reducing P-gp expression and promoting DNR-induced cell apoptosis.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors' contribution**

ZF conducted the experiments, analyzed the data, and partially wrote the manuscript. QC conducted the experiments and partially wrote the manuscript. Both were responsible for the design of this study and ensured the accuracy and completeness of the results.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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