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ORIGINAL RESEARCH

CD40L inhibits cell growth of THP-1 cells by suppressing the PI3K/Akt pathway

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Introduction: Acute myeloid leukemia (AML), the hematological malignant tumor with high mortality, is still difficult to treat. CD40L is a type II transmembrane protein, which has been reported to have the potential to inhibit growth of some cancer cells.

Materials and methods: In order to determine the role of CD40L on AML-M5 cell line THP-1, we overexpressed CD40L in the cells using a lentiviral vector system (pHBLV-CMVIE-Zs Green-T2A-puro vector); overexpression was confirmed by the detection of green fluorescent protein and CD40L protein expression.

Results: Cellular apoptosis, proliferation, and cycle assays showed that CD40L could promote the apoptosis of, suppress the proliferation of, and stimulate the arrest of the G1/S phase of THP-1 cells. Finally, the protein expression of P53, Bax/Bcl-2, cyclinD1, PCNA, PTEN, and p-Akt illustrated that CD40L may partly influence cell growth of THP-1 cells through those genes, which was confirmed by immunohistochemistry and a PI3K/Akt activator.

Conclusion: Taken together, CD40L could inhibit cell growth of THP-1 cells through the PI3K/Akt pathway, indicating that the overexpression of CD40L may be a potential target to treat the AML-M5 disease.

Keywords: CD40L, cell proliferation, AML-M5, P53, cyclinD1, PCNA, tumor suppressor, cell apoptosis

Introduction

Acute myeloid leukemia (AML) is the most common malignant hematological tumor of adults, especially the elderly.[1] AML has relatively high incidence rates, high relapse rates, and low remission rates, which has threatened human health worldwide.[2] Basic research and clinical studies on AML have recently shown progress, however, the understanding of AML is lacking, and there is still a need to find better treatments for AML.[3–5] AML has 8 subtypes (M0–M7) according to the FAB (French-American-British) classification system and one of the AML subtypes, AML-M5, is further classified into AML-M5a and M5b.[6] It has been reported that M5a and M5b have similar immunophenotypes, cytogenetics, and clinical outcomes.[7]

CD40/CD40L is suspected to be closely related to tumor formation, immunity, and inflammation.[8,9] CD40 belongs to the tumor necrosis factor receptor (TNFR) family, and CD40L (CD40 ligand, CD154), the natural ligand of CD40, belongs to the tumor necrosis factor (TNF) family.[10] It has been shown that CD40 is expressed on the surface of normal cells and cancer cells of the bladder,[11] lungs, and ovaries.[12,13] CD40 was also shown to be highly expressed in malignant hematological tumors, such as lymphocytic leukemia, lymphoma, multiple myeloma, and acute myeloid leukemia.[14] Additionally, CD40L has been demonstrated to be highly expressed in many cancers, but there is still controversy about the tumorigenic functions of CD40L in

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neoplastic disease.[15] This is because results from some studies suggest that CD40L has a tumorigenic role while those from others suggest the opposite.

There are not enough studies on the function of CD40L in AML-5M. As a result, we investigated the role of CD40L in cell growth and the PI3K/AKT pathway by overexpressing the CD40L gene in the acute monocyte leukemia cell line THP-1.[16,17]

Materials and methods

Cell culture

The human monocyte leukemia THP-1 cells used in this study were kindly provided by the Stem Cell Bank, Chinese Academy of Sciences. THP-1 cells at a concentration of 1×10^5 cells/mL were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% (v/v) penicillin/streptomycin in a 5% CO_2-humidified incubator at 37°C.

Plasmid construction and cell transfection

The full-length cDNA of human CD40L (NM_000074.2) was obtained from the National Center for biotechnology Information (NCBI) database. CD40L cDNA was inserted into the lentiviral transfer vector (pHBLV-CMVIE-Zs Green-T2A-puro vector, which was purchased from Hanhbio Co., Ltd. (Shanghai), with the restriction sites EcoR I and BamH I. Viral stocks were produced by the calcium phosphate transient transfection of 293 T cells (purchased from Hlkbio Co., Ltd., Wuhan). As a result, the assays that followed were carried out on three groups of cells: THP-1 cells, NC-LV THP-1 cells, and CD40L-LV THP-1 cells (unless otherwise stated).

Cell apoptosis

Cell apoptosis was measured by the Annexin V-FITC/PI kit (Bioswamp, Wuhan, China). The cells of each group cultured in 24-well plates were collected and 1×10^6 cells, washed twice with ice-cold PBS and then re-suspended in ice-cold binding buffer. Then, 5 μL of Annexin V-FITC and 5 μL of PI were added. The mixture was incubated for 20 minutes at 4°C in the dark, and then the cell samples were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA). We used the one-step fluorescence compensation strategy to eliminate interference with the FITC channel.

Cell cycle

Cell cycle was examined by using the cell cycle kit (Beyotime, Shanghai, China) according to the manufacturer's manual. The cells cultured in 24-well plates were harvested by centrifugation at 1,200× *g* for 5 minutes, washed twice with ice-cold PBS, and re-suspended with ice-cold 75% (v/v) ethanol. The cells were then washed with ice-cold PBS and PI was added, and the cell cycle was observed by flow cytometry (Beckman Coulter, USA).

Trypan blue staining assay

Trypan blue dye (Beyotime, China) was utilized to detect the cell death of the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells. The cells in 24-well plates were harvested and mixed with 0.4% trypan blue and counted using a hemocytometer and optical microscope. The cell viability rate (%) was calculated using the following formula: (cell number of undyed cells/total cell number) ×100.

CCK-8 assay

The Cell Counting Kit-8 assay (CCK-8, Beyotime, China) was used to identify the cell viability of the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells. The cells in 24-well plates at a concentration of 1×10^6 cells/mL were collected and 10 μL of CCK-8 solution was added to measure the OD values at a wavelength of 450 nm at 24 hours, 48 hours, and 72 hours.

Immunocytochemistry

The expression of p-Akt, P53, and cyclinD1 was observed by immunocytochemistry staining. Approximately 1×10^5 cells were cytocentrifuged onto a clean glass slide, fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.25% cold triton for 15 minutes, and incubated with 5% bovine serum albumin for 1 hour at room temperature for blocking. The primary antibodies were added and incubated overnight at 4°C. Then, the secondary antibodies were added and incubated for 1 hour at 37°C followed by incubation with diaminobenzidine (DAB) for 5 minutes at room temperature. The slides were then counterstained for 3 minutes at room temperature with hematoxylin, dehydrated for 2 minutes using gradient ethanol washes (70%, 85%, 95%, and 100%), and finally placed in xylene for 5 minutes.

The primary antibodies used were as follows: rabbit anti-human monoclonal phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (#4060, 1:400; Cell Signaling Technology Danvers, MA, USA); rabbit anti-human polyclonal anti-p53 antibody (Abcam, Cambridge, UK; ab131442, 1:100); rabbit anti-human polyclonal anti-cyclin D1 antibody [AF2G5] (ab134175, 1:100; Abcam). The secondary antibody used was goat anti-rabbit IgG H&L (HRP) antibody (ab6721, 1:1,000; Abcam).

Western blotting

The protein concentrations of CD40L in the cells of each group were detected. The protein concentrations of PTEN, p-Akt, P53, Bax, Bcl-2, CCND1, and PCNA in the cells of the three groups were also determined. We used IGF-1 as a stimulus to monitor Akt signaling. The NC-LV THP-1 and CD40L-LV THP-1 cells were treated with or without the p-Akt activator, which was 100 ng/mL of IGF-1 for 1 hour, and then the protein expression of p-Akt, P53, and Bax/Bcl-2 was detected.

Total protein was extracted and the concentrations were measured by a BCA protein assay kit (Beyotime, China). The protein was separated through SDS-PAGE gel electrophoresis and transferred onto PVDF membranes. The membranes were then blocked with 5% nonfat milk and incubated with the primary and secondary antibodies. The blots were developed with a DBA kit (Beyotime, China), and the images were acquired by gel analysis software (Quantity one, Bio-Rad, Hercules, CA, USA).

The primary and secondary antibodies used were as follows: rabbit anti-human polyclonal anti-CD40L antibody (ab65854, 1:500; Abcam); rabbit anti-human polyclonal anti-PI3 kinase p110 delta antibody (ab1678, 1:3,000; Abcam); rabbit anti-human monoclonal phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (#4060, 1:3,000 Cell Signaling Technology); rabbit anti-human polyclonal anti-p53 antibody (ab131442, 1:1,000; Abcam); rabbit anti-human monoclonal anti-Bax antibody (ab32503, 1:2,000; Abcam); rabbit anti-human monoclonal anti-Bcl-2 antibody (ab32124, 1:1,000; Abcam); rabbit anti-human polyclonal anti-cyclin D1 antibody [AF2G5] (ab134175, 1:10,000; Abcam); rabbit anti-human polyclonal anti-PTEN antibody [Y184] (ab32199, 1:10,000; Abcam); rabbit anti-human polyclonal anti-PCNA antibody [PC10] (ab29, 1:1,000; Abcam), and goat anti-rabbit IgG H&L (HRP) antibody (ab6721, 1:10,000; Abcam).

Statistical analysis

All data are presented as the mean ± standard deviation. SPSS version 19.0 software (IBM Corporation, Armonk, NY, USA) was used for the statistical analyses, and GraphPad Prism 5.0 (San Diego, CA, USA) was used to draw the figures. The cellular apoptosis, cycle, viability, death, and cloning ability quantitative data and the Western blot analysis were evaluated for statistical significance using a one-way ANOVA test. Statistical significance was established at $P<0.05$.

Results

Overexpression controls

In order to assess the efficiency of the transfection and CD40L expression of the vector in THP-1 cells, the presence of green fluorescent protein in the THP-1, NC-LV, and CD40L-LV THP-1 cells was determined by fluorescence microscopy (100×). Results in Figure 1A revealed that the green fluorescent protein was expressed with almost the same efficiency in the normal control (NC) vector and CD40L vector, while green fluorescent protein was not detected in the THP-1 cells, indicating that the vectors were successfully overexpressed in the THP-1 cells.

We evaluated the protein concentration of CD40L in the THP-1, NC-LV, and CD40L-LV THP-1 cells to further confirm the CD40L expression in the overexpression vector. The results showed that the protein expression of CD40L in the CD40L-LV THP-1 cells was significantly higher than in the THP-1 and NC-LV THP-1 cells ($P>0.05$), which verified that CD40L was successfully overexpressed in the THP-1 cells (Figure 1B).

Overexpression of CD40L suppresses THP-1 cell proliferation and induces G1/S arrest

In order to investigate the function of CD40L in the THP-1 cells, we detected the cell cycle and cell proliferation in the

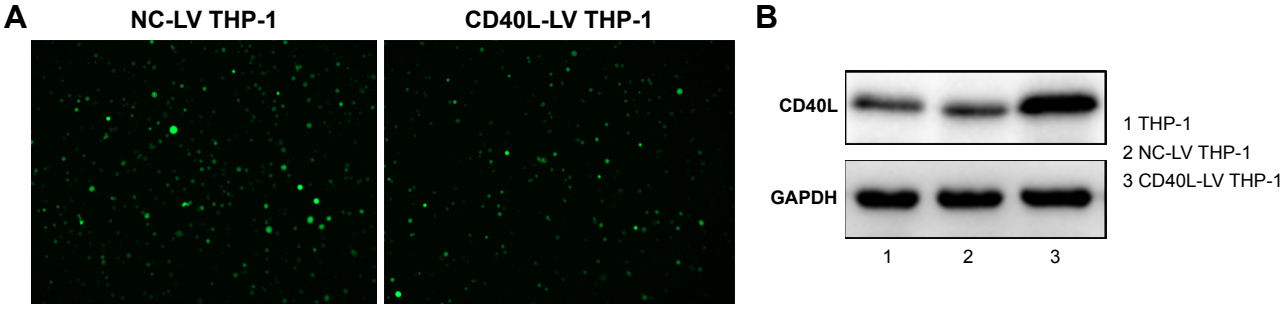

Figure 1 CD40L was overexpressed in THP-1 cells.
Notes: (**A**) The expression of the green fluorescent CD40L protein in the NC-LV THP-1 and CD40L-LV THP-1 cells. (**B**) The protein expression of CD40L in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells. Magnification 100×.

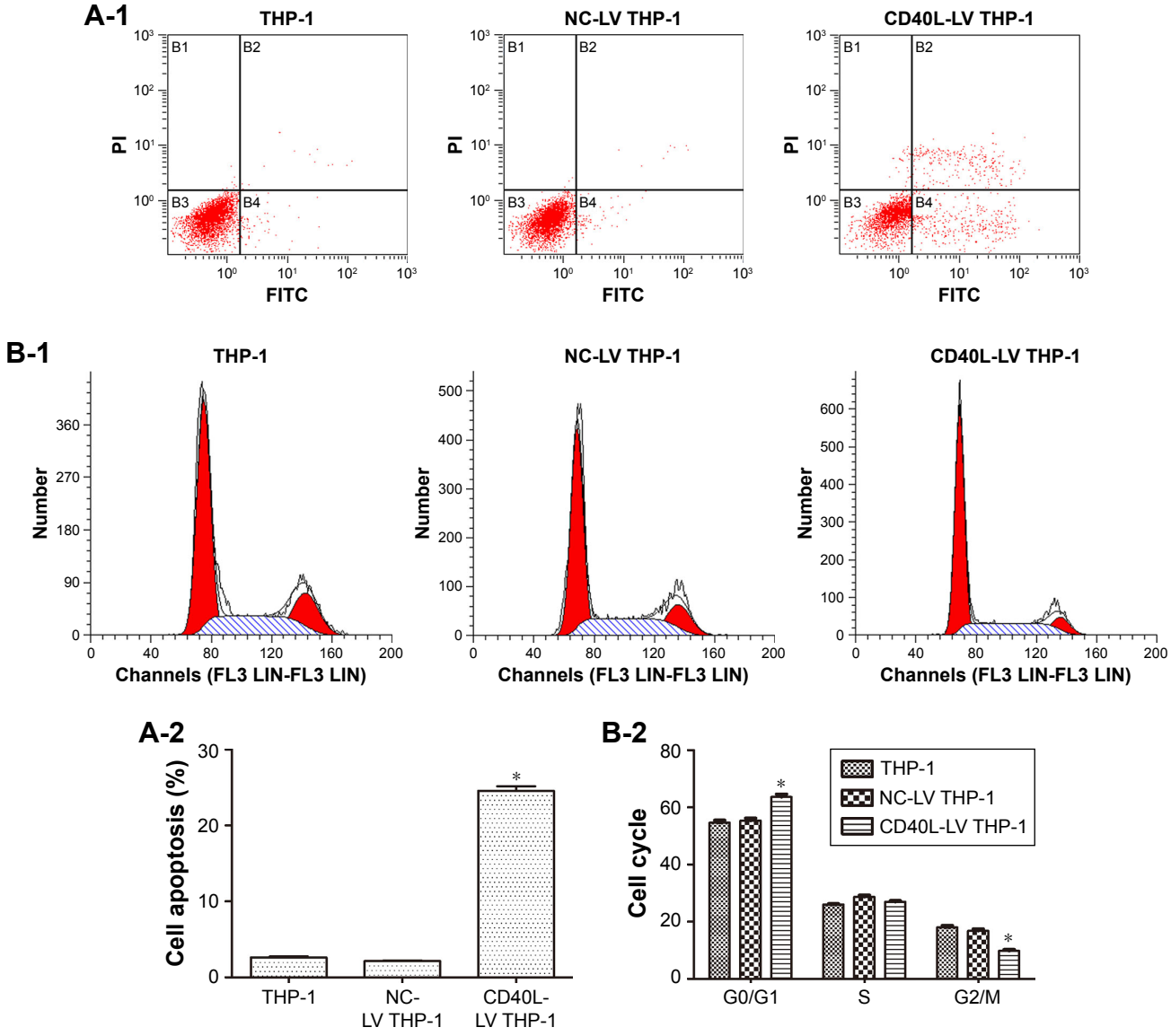

Figure 2 The impact of CD40L on the cell apoptosis and cell cycle of THP-1 cells.
Notes: (**A-1**) Apoptosis was determined by flow cytometry with Annexin V/PI staining. (**A-2**) Cell apoptosis in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells. (**B-1**) Cell cycle in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells. (**B-2**) Quantitative analysis of the cell cycle. *$P<0.05$ vs NC-LV THP-1.

three groups. The results in Figure 2B (–1, –2) indicated that the G0/G1 phase in the CD40L-LV cells was significantly higher than that in the THP-1 and NC-LV THP-1 cells ($P<0.05$) and the G2/M phase in the CD40L-LV was significantly lower than that in the THP-1 and NC-LV THP-1 cells. The results in Figure 3B showed that the cell proliferation of the CD40L-LV THP-1 cells was significantly lower than that of the other two groups ($P>0.05$).

CD40L overexpression is associated with the basal upregulation of apoptotic and non-apoptotic cell death in THP-1 cells

To determine the role of CD40L in THP-1 cell apoptosis, we measured cell apoptosis in the THP-1, NC-LV, and CD40L-LV THP-1 cells. The results in Figure 2A (–1, –2) and Figure 3A (–1, –2) revealed that cell apoptosis was higher in the CD40L-LV group than in the THP-1 and NC-LV groups ($P<0.05$), and the difference between the THP-1 and NC-LV THP-1 cells was not significant. This indicated that the CD40L overexpression was associated with the basal upregulation of apoptotic and non-apoptotic cell death in THP-1 cells.

CD40L induces the upregulation of PTEN and suppresses pro-survival Akt signaling in THP-1 cells

Because the PI3K/Akt pathway is included in the regulatory network of cell growth, we further measured the expression

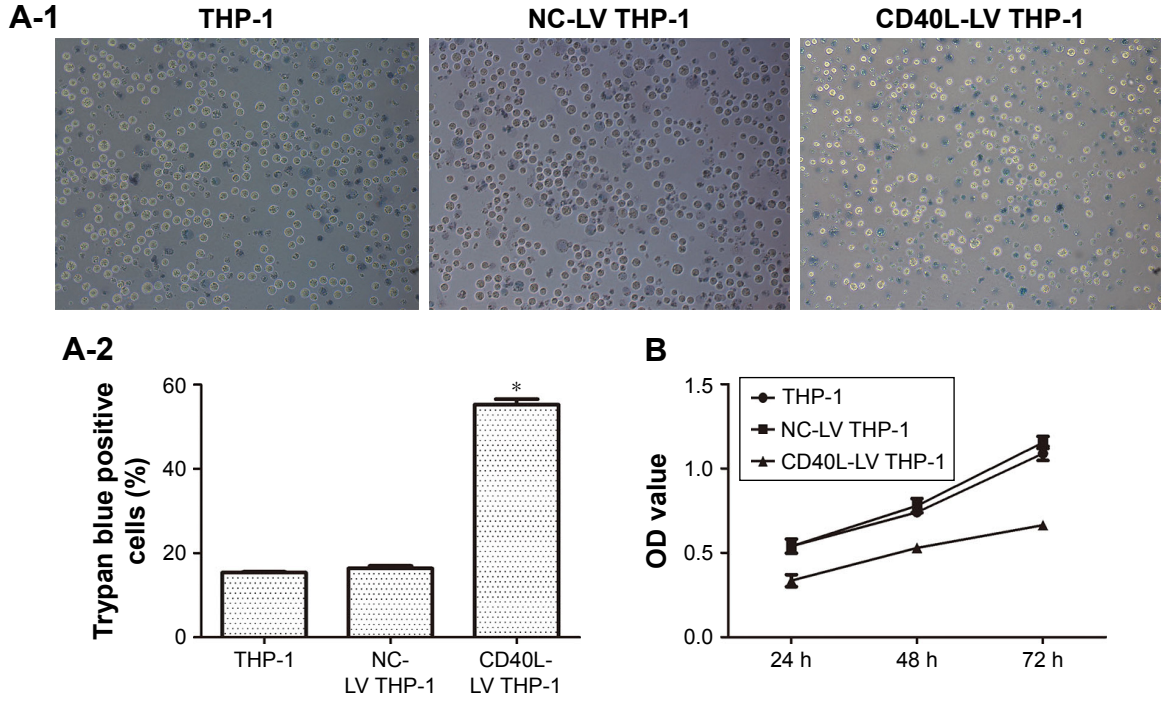

Figure 3 The impact of CD40L on the cell death and proliferation of THP-1 cells.
Notes: (**A-1**) The cell death in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells measured by trypan blue staining (200×). (**A-2**) Quantitative analysis of trypan blue positive cells. (**B**) Cell proliferation in these the three groups using the CCK-8 assay. *$P<0.05$ vs NC-LV THP-1.

levels of this pathway through Western blot and immunocytochemistry. From the results in Figure 4, we could infer that CD40L inhibited the expression of p-Akt, Bcl-2, CCND1, and PCNA, while it increased that of PTEN, P53, and Bax. The results in Figure 5 showed that CD40L suppressed the expression of p-Akt and CCND1, while it increased that of P53. We used a p-Akt activator to treat the NC-LV and CD40L-LV THP-1 cells, which revealed that CD40L regulated its downstream genes (P53, Bax/Bcl-2, cyclinD1, and PCNA) through the PI3K/Akt pathway (Figure 6).

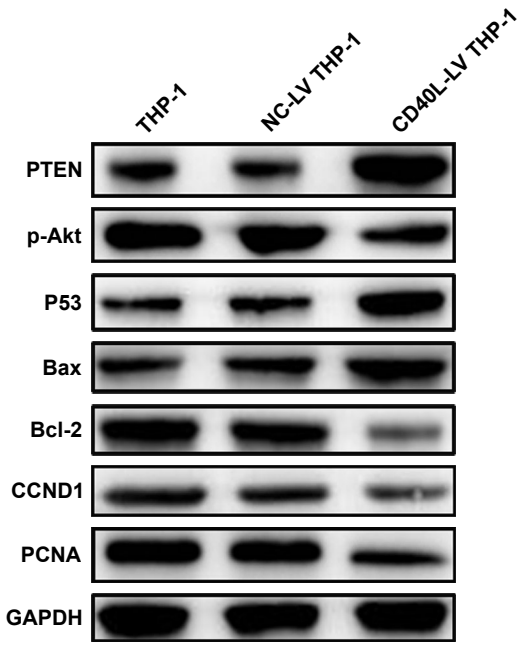

Figure 4 The impact of CD40L on the protein expression of the PI3K/Akt pathway.
Note: Protein expression levels in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells were determined by Western blotting.

Discussion

Despite the signs of progress on the molecular mechanisms of AML, there is still a lack of effective treatments for AML patients. CD40L, a 33 kDa type II transmembrane protein, was revealed to participate in many cancers and inflammations. But the function of CD40L in different cancers has been inconsistent. In lung cancer cells and pancreatic cancer,[12,18] CD40L had anti-cancer effects. In contrast, in multiple myeloma and chronic lymphocytic leukemia,[19,20] CD40L improved the survival of tumor cells through the up-regulation of the vascular endothelial growth factor (VEGF). Because few studies have investigated the role of CD40L in AML-M5, we explored its function in the AML-M5 cell line THP-1.

CD40L has been noted to induce cell apoptosis in pancreatic cancer cell lines.[18] In our study, the results suggested that CD40L could also induce the cell apoptosis of THP-1 cells. In human breast cancer cells, the expression of cyclinD1 (CCND1) was suppressed when the cell cycle was arrested in the G0/G1 phase.[21] In our study, CD40L

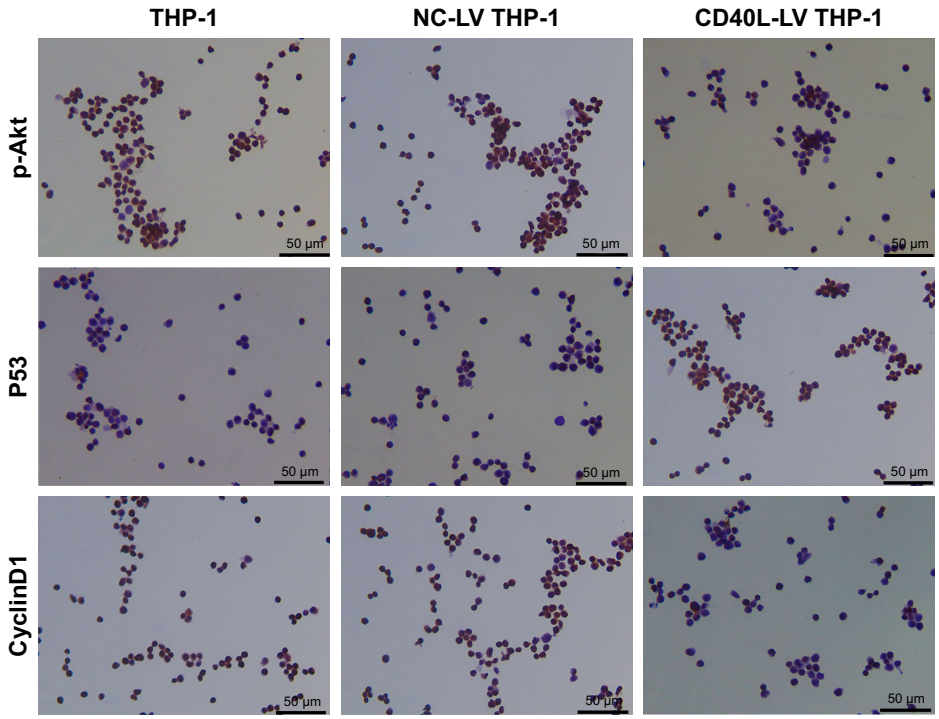

Figure 5 The expression of p-Akt, P53, and cyclinD1 detected by immunohistochemistry.
Note: The expression of p-Akt, P53, and cyclinD1 in the THP-1, NC-LV THP-1, and CD40L-LV THP-1 cells was detected by immunohistochemistry (400×).

might have also induced G0/G1 arrest by suppressing the expression of cyclinD1 genes. CD40L has been shown to induce cell death in bladder carcinoma cell lines.[22] Our study showed the same results. That is, CD40L inhibited the cell death of THP-1 cells. The proliferation cell nuclear antigen (PCNA) participates in DNA replication and can establish cell proliferation in both normal cells and cancer cells.[23] From the results of the CCK-8 assay and detection of PCNA expression, we could deduce that CD40L might suppress the cell proliferation of THP-1 cells by reducing PCNA expression. The PI3K/Akt signaling pathway is closely related to cell growth and cell proliferation. Activated PI3K catalyzes the conversion of PIP2 to PIP3 leading to the phosphorylation of Akt. Phosphorylated Akt can inhibit cell apoptosis and promote cell growth and proliferation. PTEN, as the negative regulator of the PI3K/Akt pathway, can alter PIP3 to PIP2.[24] The results of our study showed that CD40L could increase the expression of PTEN and inhibit the expression of p-Akt. On the basis of these data, we concluded that CD40L may suppress cell growth through the PI3K/Akt pathway.

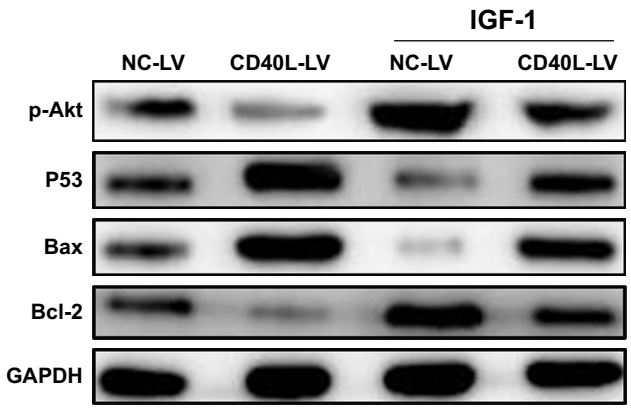

Figure 6 The protein expression of p-Akt, P53, and Bax/Bcl-2 when treated with IGF-1.
Note: The protein expression of p-Akt, P53, and Bax/Bcl-2 in the NC-LV THP-1 and CD40L-LV THP-1 cells treated with or without 100 ng/mL IGF-1 for 1 hour was determined by Western blotting.

Taken together, our current research has suggested that CD40L may be a potential tumor suppressor that regulates the expression of the P53, Bax, Bcl-2, CCND1, PCNA, PTEN, and Akt genes and suppresses the cell growth and proliferation of THP-1 cells. This finding suggests that CD40L may be a possible target to treat the AML-5M disease.

Conclusion

CD40L may be a potential tumor suppressor of THP-1 cells, which implies that CD40L may be a possible target to treat the AML-5M disease.

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Disclosure

The authors report no conflicts of interest in this work.

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