miR-124-3p enhances dendritic cell-mediated anti-tumor immunity by targeting CYLD/4-1BBL pathway

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Abstract

Background: MicroRNAs play important roles in dendritic cell (DCs)-mediated immunity, but their specific functions in lung cancer remain unclear.

Objective: To investigate miR-124-3p regulation of DC-mediated immune response via CYLD/4-1BBL pathway, and the inhibitory effect of miR-124-3p on lung cancer.

Methods: DCs were cultured and amplified in vitro, and then transfected with miR-124-3p mimic, miR-124-3P inhibitor, or siRNA CYLD. Double luciferase reporter genes were used to detect the target relationship between miR-124-3p and CYLD. qRT-PCR and western blotting were used to detect the expression levels of CYLD and 4-1BBL. Flow cytometry was used to assess the proliferation rate of $CD4^+$ T cells co-cultured with untransfected DCs and those transfected with miR-124-3p mimic or miR-124-3p inhibitor. C57BL/6 tumor bearing mice, implanted with LL/2 lung adenocarcinoma cells, were administered DCs transfected with the miR-124-3p mimic or untransfected DCs. The tumor size and weight of mice were then measured. **Results:** miR-124-3p and CYLD3'UTR contained target-binding site, and overexpression of miR-124-3p enhanced the expression of CYLD and 4-1BBL. $CD4^+$ T cells co-cultured with miR-124-3p mimic-transfected DCs showed significantly increased proliferation. In tumor-bearing mice, tumor inhibition rate was 73.5%, and tumor volume and weight were significantly decreased after the administration of DCs containing the miR-124-3p mimic.

Conclusions: The expression of CYLD was regulated by miR-124-3p, which, in turn, increased the expression of 4-1BBL. miR-124-3p regulated DCs function via CYLD/4-1BBL cascade. miR-124-3p plays important roles in DCs-induced T cells, thereby enhancing anti-tumor immunity.

Introduction

MicroRNAs (miRNAs) post-transcriptionally regulate gene expression by interacting with the untranslated region (UTR) of the target gene *(1)*. The contributions of miRNAs to the functions of the immune system remain largely undetermined. Among them, miR-124 plays the role of tumor suppress or gene in a variety of tumors. Previous studies have shown that the expression of miR-124 is significantly higher in CD24+cDC1 cells than in pDCs and CD172α + cDC2 cells *(2)*, indicating that miR-124-3p may be involved in DC development. CYLD lysine 63 deubiquitinase (CYLD) is a deubiquitinating enzyme that removes lysine 63-linked polyubiquitin chains from target proteins, and regulates cellular survival and proliferation. CYLD is widely distributed in human body; defects in CYLD or its deletion can lead to cutaneous tumors of the head and face, such as familial cylindroma. CYLD is also involved in the regulation of various immune-cell functions *(3)*, plays important roles in tumor immunity, and can directly regulate the levels of 4-1BBL in DCs. 4-1BB/4-1BBL (CD137/CD137L), a member of the tumor necrosis factor receptor superfamily 9, is a potential target in immune therapy. As an activated receptor on the surface of DCs and T cells, it can enhance the generation of an effective tumor-specific immune response in situ *(4)*. The purpose of this study was to demonstrate the existence of a CYLD/4-1BBL signaling axis and determine

its role in DC-mediated anti-tumor immunity. The results obtained in our present study will help to further delineate the tumor microenvironment, functions of antigen presentation, and induction of T-cell—mediated immune response. These findings will improve the efficacy of targeted anti-tumor immunotherapies, thereby benefitting patients with cancer.

Results

Effect of miR-124-3p on expression of CYLD and 4-1BBL in DCs

qRT-PCR was used to investigate whether miR-124-3p could regulate the expression of CYLD and 4-1BBL. Our results show that the relative expression levels of CYLD in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC (detailed sequences please refer to Table 1), and control groups were 3.182±0.212, 0.635±0.102, 1.423±0.165, 1.320±0.152, and 1.372±0.213, respectively (Fig.1A). The relative expression of CYLD in the miR-124-3p mimic group was significantly higher than that in the miR-124-3p inhibitor and control groups (P<0.05); the relative expression of CYLD in the miR-124-3p inhibitor group was lower than that in control group (P<0.05). The relative expression levels of 4-1BBL in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC, and control groups were 4.812±1.164, 0.642±0.128, 1.181±0.132, 1.153±0.243, and 0.994±0.227, respectively (Fig.1B); no statistically significant differences were observed in the relative expression levels of 4-1BBL between miR-124-3p mimic, miR-124-3p inhibitor, and the control group (P<0.05). The relative expression of 4-1BBL in the miR-124-3p mimic group was significantly higher than that in the miR-124-3p inhibitor and control groups (P<0.05), while the relative expression of CYLD in the miR-124-3p inhibitor group was lower than that in the control group (P<0.05). Western blotting analysis showed that the expression levels of CYLD protein in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC, and control groups were 206.33±12.32, 54.23±9.75, 99.47±3.52, 96.52±4.28, and 97.26±4.15, respectively (Fig.1C). The expression levels of 4-1BBL protein in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC, and the control groups were 202.35±21.66, 55.89±8.48, 102.75±5.57, 98.79±6.24, and 99.22±6.32, respectively (Fig.1D). There was a statistically significant difference in the expression of CYLD and 4-1BBL proteins among the miR-124-3p mimic, miR-124-3p inhibitor, and the control group (P<0.05) (Fig.1E).

Table1. microRNA-124 mimic and inhibitor sequences

Names	Sequences(5'–3')
miR-124 mimic	UAAGGCACGCGGUGAAUGCC
miR-124 inhibitor	GGCAUUCACCGCGUGCCUUA
Inhibitor NC	CAGUACUUUUGUGUAGUACAA
Mimic NC	UUGUACUACACAAAAGUACUG

We used the Pearson correlation coefficient to analyze the correlations between the expression of CYLD and that of 4-1BBL in DCs. The results of correlation analysis showed a positive correlation between the

relative expression of CYLD and that of 4-1BBL (r=0.865, *P*<0.001). Correlations between CYLD and 4-1BBL expression were determined using western blotting (r=0.855, *P*<0.001).

Ability to stimulate 4-1BBL expression is impaired in CYLD-knockdown DCs

To investigate whether CYLD knockdown affected the expression of 4-1BBL in DCs, we co-transfected DCs with CYLD siRNA (100nM for 48h), and with miR-124-3p mimic or miR-124-3p inhibitor; DCs transfected with CYLD siRNA only were designated as the control group. The relative expression levels of 4-1BBL in siRNA CYLD group, miR-124-3p mimic + siRNA CYLD group, and miR-124-3p inhibitor + siRNA CYLD group were 0.22±0.17, 0.24±0.15, and 0.18±0.12, respectively, as assessed using qRT-PCR (Fig.2A). There was no statistically significant difference in the relative expression levels of 4-1BBL among these three groups. Western blotting analysis indicated that the expression levels of 4-1BBL in siRNA CYLD group, miR-124-3p mimic + siRNA CYLD group, and miR-124-3p inhibitor + siRNA CYLD group were 23.84±6.27, 24.66±5.21, and 20.73±2.54, respectively (Figs.2B,2C). The efficiency of siRNA-mediated CYLD knockdown was assessed using qRT-PCR and western blotting, which confirmed that miR-124-3p lost the ability to stimulate the expression of 4-1BBL in CYLD-knockdown DCs. Taken together, these results indicate that the expression of 4-1BBL was regulated upstream by CYLD rather than miR-124-3p.

Expression of miR-124-3p by DCs affects the proliferation of $CD4^+$ T cells

To investigate the stimulating effect of DCs transfected with miR-124-3p mimic or miR-124-3p inhibitor, and that of untransfected DCs, on $CD4^+$ T cells, CFSE-labeled $CD4^+$ T cells were co-cultured with each group of DCs for 72hours; then, flow cytometry was used to detect the proliferation of these $CD4^+$ T cells (Fig.3). The percentage of CD4+ T cells in miR-124-3p mimic group was greater than that in miR-124-3p inhibitor group and in untransfected DCs group. The percentages of $CD4^+$ T cells differed significantly among these three groups (*P*<0.05), the results indicate that DCs transfected with miR-124-3p mimic significantly promoted the proliferation of $CD4^+$ T cells.

Verification of targeting relationship between miR-124-3p and *CYLD*

We predict the existence of binding sites between miR-124-3p and *CYLD*3'UTR from the on-line website tool: http://www.targetscan.org/, suggesting that *CYLD* may be a direct target of miR-124-3p (Fig.4). The results of dual luciferase reporter assay showed that miR-124-3p mimic inhibited the luciferase activity of *CYLD-WT* reporter gene (*P*<0.05) but exerted no significant effect on that of *CYLD-MUT* (Table2).

Table2. Detection of luciferase activity

miR-124-3p	CYLD	
WT	MUT	
Mimic NC	1.00±0.11	1.03±0.18
Mimic	0.38±0.04	0.98±0.10
t	9.175	0.421
p	0.001	0.696

Tumor inhibition rate

Lewis lung cancer cells (LL/2) were inoculated into C57BL/6 mice, and tumors were detectable at approximately 15 days post-inoculation. On day 16 post-inoculation, tumor volume was measured, and then the mice in each group were injected with miR-124-3p mimic-transfected DCs, siRNA CYLD-transfected DCs, or untransfected DCs via the tail vein once every 3 days, four times altogether. At approximately 4 weeks post-injection, no mortality was recorded in found any of these groups of mice. The mice were sacrificed by cervical dislocation (Fig.5A). The tumors were dissected and weighed, and the length and width of each tumor was measured (Fig.5B). Our results showed the tumor volume in miR-124-3p mimic-transfected DCs group, siRNA CYLD-transfected DCs group, and untransfected DCs group to be 121.18±29.67, 1326.42±49.76, and 1022.15±56.37mm^3, respectively. These results indicate that compared with the mice administered untransfected DCs, mice administered siRNA CYLD-transfected DCs showed the most rapid tumor-growth rate, while the mice administered miR-124-3p mimic-transfected DCs showed the slowest growth rate and smallest tumor volume (Figs.5C and 5D). Tumor inhibition rate was 73.5%, and there were significant differences in tumor weight and volume among the three groups (P<0.05). These results suggest that miR-124-3p inhibited the growth of lung tumors by targeting CYLD in vivo.

Discussion

Non-small cell lung cancer is one of the most common malignancies worldwide. Patients with early lung cancer can be asymptomatic and are, therefore, diagnosed in middle and late stages of the disease. For that reason, therapeutic efficacy and prognosis are poor in this patient population *(5)*. While the recently developed immunotherapies against lung cancer show promise, some of the patients still do not benefit from these new treatment approaches, highlighting the importance of delineating the immune mechanisms of lung cancer. DCs are powerful professional antigen-presenting cells that can identify and process tumor antigens, and activate specific T-cell responses to tumors, which is key in tumor immunity *(6)*. The immunesuppressive internal environment in the tumor body can lead to abnormal differentiation of DCs precursors. Concurrently, immunosuppressive factors, such as VEGF, TGF-β, and IL-10, secreted by tumor cells, also hinder DCs maturation. This process results in generation of DCs that lack the ability to recognize and present tumor antigens, and have difficulty activating T cells and inducing an immune

response. This cascade aids the tumor in its immune escape *(7)*. For these reasons, investigating tumor-infiltrating DCs will help reveal the mechanisms of tumor immune escape. In filtrating myeloid dendritic cells (mDCs) are good predictors of tumor prognosis. By regulating immunosuppressive factors in the tumor microenvironment or targeting the genes regulating DCs maturation, mDCs can effectively promote maturation and activate the immune response with increased efficiency *(8)*. DCs play a key role in tumor immunotherapy, and relevant research is ongoing. CYLD is a deubiquitination enzyme that regulates various signaling pathways in physiological processes *(9)*. Our previous studies on the distribution and differential expression of CYLD and DCs in lung cancer tissues and corresponding adjacent tissues showed that CYLD expression is correlated with the distribution of mDCs in lung cancer and adjacent tissues. Those studies showed that increased expression levels of the CYLD protein in lung-cancer tissues are correlated with increased rate of DCs maturation *(9).*

miRNAs play important roles in regulating gene expression in tumor biology *(10)*. Studies have shown that the expression of miR-124 in $CD24^+$ cDC1 cells is significantly higher than that in pDCs and $CD172\alpha^+$ cDC2. These results suggest that miR-124 may be involved in the development of DCs subsets *(2)*. miR-124-3p plays various roles in tumor suppression, and its expression is downregulated in different types of cancer *(11-13)*. In this study, we used bioinformatics analysis to predict the existence of a binding site between miR-124-3p and CYLD3'UTR. The results of our dual luciferase assay confirmed that *CYLD* was the target of miR-124-3p. Next, we transfected miR-124-3p mimic and miR-124-3p inhibitor into DCs to assess their levels of CYLD expression. Our results show that the expression of CYLD in DCs was positively regulated by miR-124-3p. Concurrently, the expression of CYLD was also positively correlated with that of 4-1BBL. Blocking the expression of CYLD abrogated miR-124-3p-mediated regulation of 4-1BBL expression. These results suggest that miR-124-3p regulates the expression of CYLD and then 4-1BBL. These findings confirm the existence of a miR-124-3p/CYLD/4-1BBL signaling pathway. Our previous study showed that CYLD can inhibit tumor-cell proliferation by blocking the Bcl-3-dependent NF-κB pathway. Downregulation of NF-κB expression can protect DCs, promote their maturation, and influence the DCs-mediated immune response. Upregulation of CYLD expression in lung adenocarcinoma can enhance the DCs-mediated anti-tumor effect. Other studies have also shown that CYLD can regulate cancer-cell apoptosis via deubiquitination of multiple signaling pathways such as those involving the activity of TGF-β-and NF-kB *(15)*. Studies have shown the existence of a reverse signal-transduction pathway between NF-kB and 4-1BBL, and reverse signal transduction through 4-1BBL is related to the nuclear translocation of NF-kB *(16)*. Therefore, CYLD may be associated with 4-1BBL via NF-kB signaling. However, the exact mechanism of CYLD-mediated regulation of 4-1BBL expression remains to be determined.

In our previous study, we found that upregulation of CYLD expression can enhance DCs-induced T-cell proliferation, thereby mediating immune regulation in lung adenocarcinoma *(14)*. We found that decreased nuclear translocation of CYLD can regulate DCs differentiation and the proliferation and function of $CD4^+$ T cells *(17)*. Our results indicated that *CYLD*-knockout DCs show decreased signaling and nuclear translocation of NF-kB. Inhibition of the NF-kB pathway inhibits the DCs-induced proliferation

and function of $CD4^+$ T cells, indicating that CYLD exerts a positive effect on T-cell function *(9)*. In this study, DCs were transfected with a miR-124-3p mimic or miR-124-3p inhibitor, and were then co-cultured with $CD4^+$ T cells. The results of our ELISA immune assay showed that secretion of IFN-γ and IL-17 by $CD4^+$ T cells was significantly increased, indicating that miR-124-3p not only upregulated CYLD expression, but also participated in the induction of T-cell mediated immune response.

4-1BB/4-1BBL is a major costimulatory molecule. The signaling enabling interactions between 4-1BB and 4-1BBL is transmitted into cells via activated NF-kB and the TRAF2-NIK pathway; these events stimulate T cells to secrete IL-2 and promote the proliferation of activated T cells *(18-20)*. 4-1BB/4-1BBL and CD28/B7 are costimulatory molecules that play important roles in organ transplantation, tumor therapy, and autoimmune diseases *(21)*. Studies have shown that 4-1BB can synergistically stimulate the activation and proliferation of $CD4^+$ T and $CD8^+$ T cells. While 4-1BB plays an important role in the activation and proliferation of $CD8^+$ T cells, CD28 signaling is more conducive to the activation of $CD4^+$ T cells *(22)*. Habib-Agahi noted that 4-1BBL can promote CD28 expression in activated T cells *(23)*.

4-1BBL is expressed on the surface of various activated antigen-presenting cells (APC), such as IFN-γ activated macrophages, CD40 ligand-activated B cells, monocytes, and DCs. However, recent studies have shown that 4-1BBL is also expressed on the surface of certain tumor cells. Additionally, 4-1BB/4-1BBL expression is closely related to the occurrence and development of tumors, and to tumor prognosis *(24)*. As an immune costimulatory molecule, 4-1BB/4-1BBL can enhance a tumor-specific immune response in situ. We introduced a miR-124-3p mimic or inhibitor into DCs derived from mouse bone marrow, and then injected these transfected DCs into tumor-bearing mice. Our results show that miR-124 could induce tumor shrinkage, which agreed with previous findings showing that miR-124 demonstrates tumor-inhibiting activity *(25)*.

In summary, herein we investigated the mechanisms involved in miR-124-3p-mediated regulation of lung cancer. miR-124-3p positively regulated the expression of CYLD in DCs, which, in turn, regulated 4-1BBL expression. 4-1BBL can activate T cells, thereby exerting an inhibitory effect on lung-cancer cells. In-depth understanding of CYLD/4-1BBL signaling mechanisms, mediated by miR-124-3p, will help to uncover new targets for tumor prevention and treatment.

Material And Methods

Statement for the use of experimental animals

(i) All experiments were approved by the Institutional Animal Care and Use Committee of the Provincial Clinical College of Fujian Medical University, Fujian Provincial Hospital;

(ii) The authors confirmed that all experiments were performed in accordance with relevant guidelines and regulations;

(iii) The relevant study in this work is reported in accordance with ARRIVE guidelines.

In vitro and in vivo models

The Lewis lung carcinoma cell line (LL/2), obtained from the Shanghai Enzyme Linked Biotechnology Co. Ltd., was cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Female C57BL/6 mice (6- to 8-weeks old) were purchased from Shanghai SLAC Laboratory Animal Co.,Ltd. All the mice were allowed to adapt for 1 week in a specific-pathogen-free animal room at 25°C and 60% relative humidity, using a 12 hours light/dark cycle. Artificial feed was sterilized using ^{60}Co and supplied daily. Drinking water was subjected to four-level filtration, and treated using ozone and ultraviolet (UV) radiation.

DCs isolation and culture

C57BL/6 mice were sacrificed by cervical dislocation. The femurs were separated aseptically, after which the bone marrow was harvested and washed repeatedly with RPMI 1640 medium. After centrifugation at 600×g, single bone-marrow cells were resuspended in RPMI 1640 medium. This cell suspension was seeded in a 6-well plate with RPMI 1640 containing10 ng/mL recombinant murine granulocyte macrophage colonystimulating factor (mGM-CSF; Endogene) and 10 ng/mL recombinant murine interleukin (IL) -4 (mIL-4; Endogene) at a density of 1×10^6 cells/ml. The media were changed every 2 days. After 6 days of culture, suspensions or loose adherent cells (immature BMDCs) were collected for subsequent experiments.

Establishment of Lewis lung carcinoma LL/2 cell line in C57BL/6 mice

To establish a mouse model of lung cancer, LL/2 cells in logarithmic growth phase were inoculated subcutaneously into the right side of the ribcage, using a dose of 1×10^6 cells per mouse. The inoculated mice were divided into three groups with five mice per group (n=5). Tumor volume in the mice was measured on day 16 after they were inoculated with the tumor cells. Then, the tumor-bearing mice in the three groups were injected into the tail vein with miR-124-3p mimic-transfected DCs, siRNA CYLD-transfected DCs, or untransfected DCs, respectively, every 3 days, 4 times altogether. Tumor size was measured every 3 days. After 4 weeks, the mice were sacrificed by cervical dislocation and dissected. Tumor weight and volume were measured using the formula: $(a\times b^2)/2$, where a and b are the length and width of tumor, respectively. A growth curve of the transplanted tumor was then generated, and tumor inhibition rate was calculated for each group as follows: tumor inhibition rate= [(average tumor weight of control group – average tumor weight of experimental group/average tumor weight of control group)] ×100%.

Cell grouping

DCs were randomly divided into eight groups, with untransfected DCs serving as the control group. The groups, co-transfected using miR-124-3p mimic or miR-124-3p inhibitor combined with siRNA CYLD, included: miR-124-3p mimic group; miR-124-3p inhibitor group; miR-124-3p mimic NC group; miR-124-3p

inhibitor NC group; siRNA CYLD group; miR-124-3p mimic + siRNA CYLD group; miR-124-3p inhibitor + siRNA CYLD group; miR-124-3p mimic + siRNA CYLD group; miR-124-3p inhibitor + siRNA CYLD group.

Cell transfection

miR-124-3p mimic, miR-124-3p inhibitor, and the corresponding negative controls mimic NC and inhibit or NC (Shanghai Gema Corporation) were transfected into DCs using Lipofectamine™ 2000 (Invitrogen) per manufacturer's instructions. DCs were also transfected with siRNA CYLD (siCYLD, GeneChem, China) per manufacturer's protocol. Cells were harvested at 48 hours post-transfection and subjected to RNA and protein extraction.

$CD4^+$ T cell and DCs co-culture in vitro

$CD4^+$ T cells were obtained from mouse splenocytes using immunomagnetic beads. Freshly isolated $CD4^+$ T cells were mixed with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, USA) at a final concentration of 5 μM. After incubating at 37°C for 10 min, the cells were washed with 10ml RPMI-1640 complete medium and centrifuged at 600×g for 10 min; This step was repeated three times, after which the cells were re-suspended in RPMI-1640 complete medium. For the co-culture study, the density of CFSE-labeled $CD4^+$ T cells was adjusted to 2×10^6 cells/ml. The final density of DCs transfected with miR-124-3p mimic or miR-124-3p inhibitor, and that of untransfected DCs, was adjusted to 2×10^5 cells/ml. $CD4^+$ T cells were then co-cultured with DCs in 2ml RPMI 1640 supplemented with 10% FBS for 72hours at 7°C. After 72 hours, cells were harvested, and flow cytometry was used to detect the proliferation rate of $CD4^+$ T cells.

Flow cytometry

Flow cytometry was used to detect the proliferation rate of $CD4^+$ T cells. Cells co-cultured for 72 hours were collected, centrifuged, washed, and resuspended in 500μl PBS containing 1% paraformaldehyde. Each sample of 20,000 cells was analyzed by FC500 flow cytometer (Beckman Coulter, USA). CFSE fluorescence was collected using an FL1 detection channel (525nm). CXP software was used to analyze data, and the percentage of divided cells in each group was obtained.

Dual-luciferase reporter assay

Recombinant plasmids containing a luciferase reporter gene vector for wild type pGL-WT-CYLD and mutant pGL-MUT-CYLD were constructed and sequenced normally. DCs were seeded into a 24-well plate and cultured overnight at 37°C. miR-124-3p mimic or its negative control was co-transfected together with wild-type or mutant reporter gene recombinant plasmid. After 48 hours of transfection, the activities of firefly and Renilla luciferases were detected using Dual Luciferase Reporter Gene Assay Kit per manufacturer's protocol, and expression ratio of the two luciferases was designated as relative luciferase activity. Using this ratio, we then assessed whether miR-124-3p exerted direct regulatory effects on CYLD expression.

qRT-PCR

Total RNA extraction and reverse transcription were performed in accordance with the instructions of kit manufacturers. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using PrimeScript RT Reagent Kit and SYBR Premix Ex Taq kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan). PCR cycling parameters were as follows: 95°C for 20 seconds, 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. All reactions were performed in triplicate using a 10-ml reaction volume. Values were normalized with respect to the expression of *U6* and *GAPDH*, used as housekeeping genes, and gene expression levels were calculated using the 2-dCt method. Primers used for real-time PCR were designed using Primer Express 3.0 and synthesized by Invitrogen.

Western blotting

A total of 20mg protein was used for western blotting. Samples were subjected to gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked using 5% skim milk in TBST, and incubated with primary antibodies specific for CYLD (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase- (anti-mouse IgG and anti-rabbit IgG) were used to detect the primary antibodies (Vector Labs, USA). An ECL chemiluminescence kit (CWBIO) was used for HRP detection. Protein quantity was detected using GAPDH as loading control.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. Data are expressed as mean ± SD. Student's *t*-test was used for comparison between two groups, and one-way analysis of variance (ANOVA) were used for pairwise comparison. *P* value less than 0.05 was considered statistically significant.

Declarations

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Figures

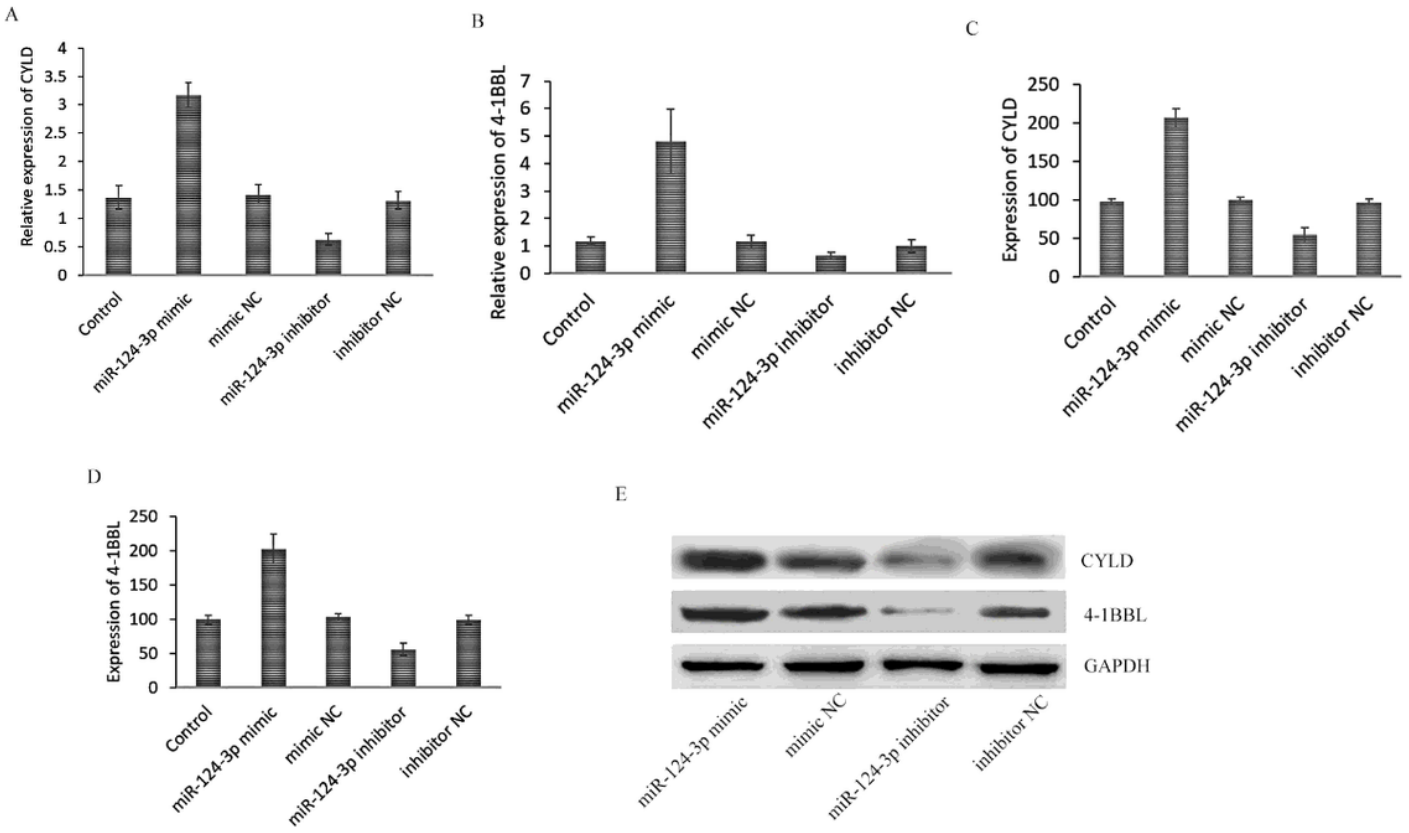

Figure 1

Relative expression of CYLD and 4-1BBL in transfected DCs. (A)Relative expression of CYLD, as determined by qRT-PCR, in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC, and control groups. (B)Relative expression of 4-1BBL, as determined by qRT-PCR, in the miR-124-3p mimic, miR-124-3p inhibitor, mimic NC, inhibitor NC, and control groups. Results of qRT-PCR, showing that relative expression of CYLD in miR-124-3p mimic group is significantly higher than that in the miR-124-3p inhibitor and miR-control groups, while that in the miR-124-3p inhibitor group is lower than that in the miR-control group. *P<0.05. (C)Expression of CYLD protein, as determined by western blotting, in miR-124-3p mimic, miR-124-3p inhibitor, mimics NC, inhibitor NC, and control groups. (D)Expression of 4-1BBL protein, as determined by western blotting, in miR-124-3p mimic, miR-124-3p inhibitor, mimics NC, inhibitor NC, and control groups. (E)Expression of 4-1BBL protein in the miR-124-3p mimic group is

significantly lower than that in the miR-124-3p inhibitor and miR-control groups, while that in the miR-124-3p inhibitor group is higher than that in the miR-control group. P<0.05.

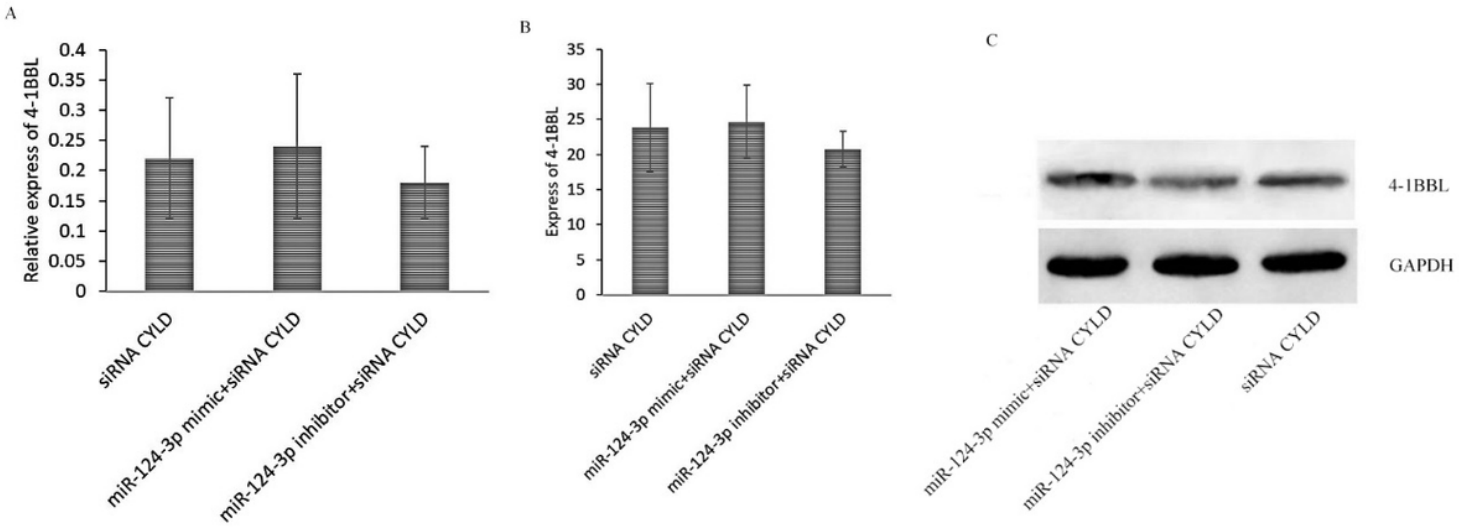

Figure 2

(A) Relative expression levels of 4-1BBL, as determined by qRT-PCR, in siRNA CYLD group, miR-124-3p mimic + siRNA CYLD group, and miR-124-3p inhibitor + siRNA CYLD group. (B, C) Expression level of 4-1BBL, as determined by western blotting, in siRNA CYLD group, miR-124-3p mimic + siRNA CYLD group, and miR-124-3p inhibitor + siRNA CYLD group. There was no statistically significant difference among these groups.*P>0.05.

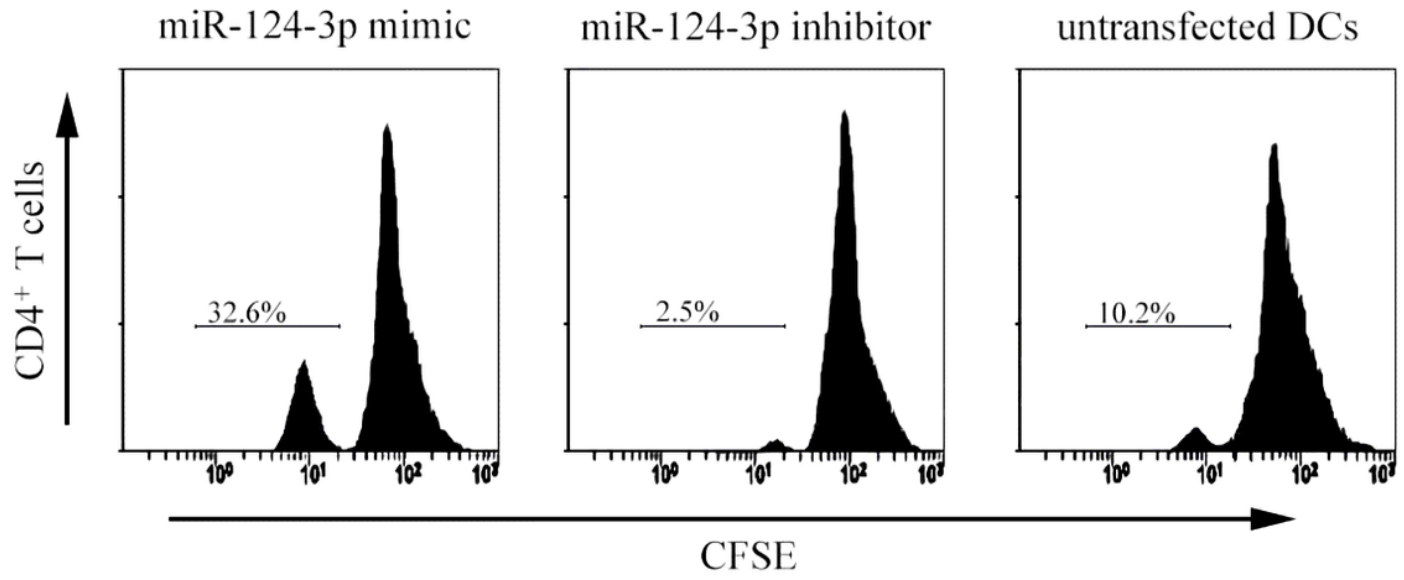

Figure 3

CFSE-mediated detection of CD4+ T-cell proliferation. CFSE-labeled CD4+ T cells were co-cultured with miR-124-3p mimic transfected DCs, miR-124-3p inhibitor transfected DCs, and untransfected DCs. The percentage of CD4+ T cells in each group was statistically significant (P<0.05).

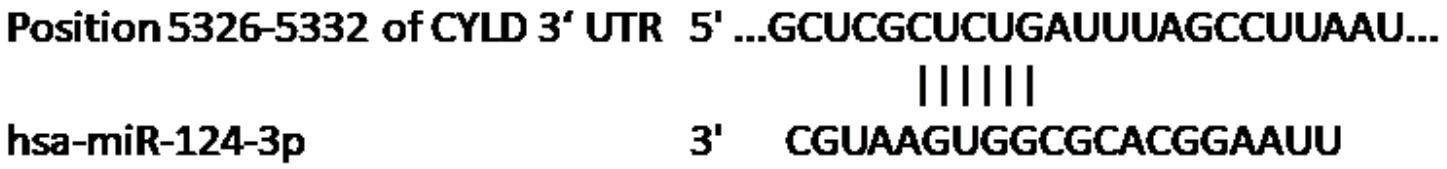

Figure 4

The potential binding sites of miR-124-3p and CYLD was conducted from theo n-line website tool: http://www.targetscan.org/

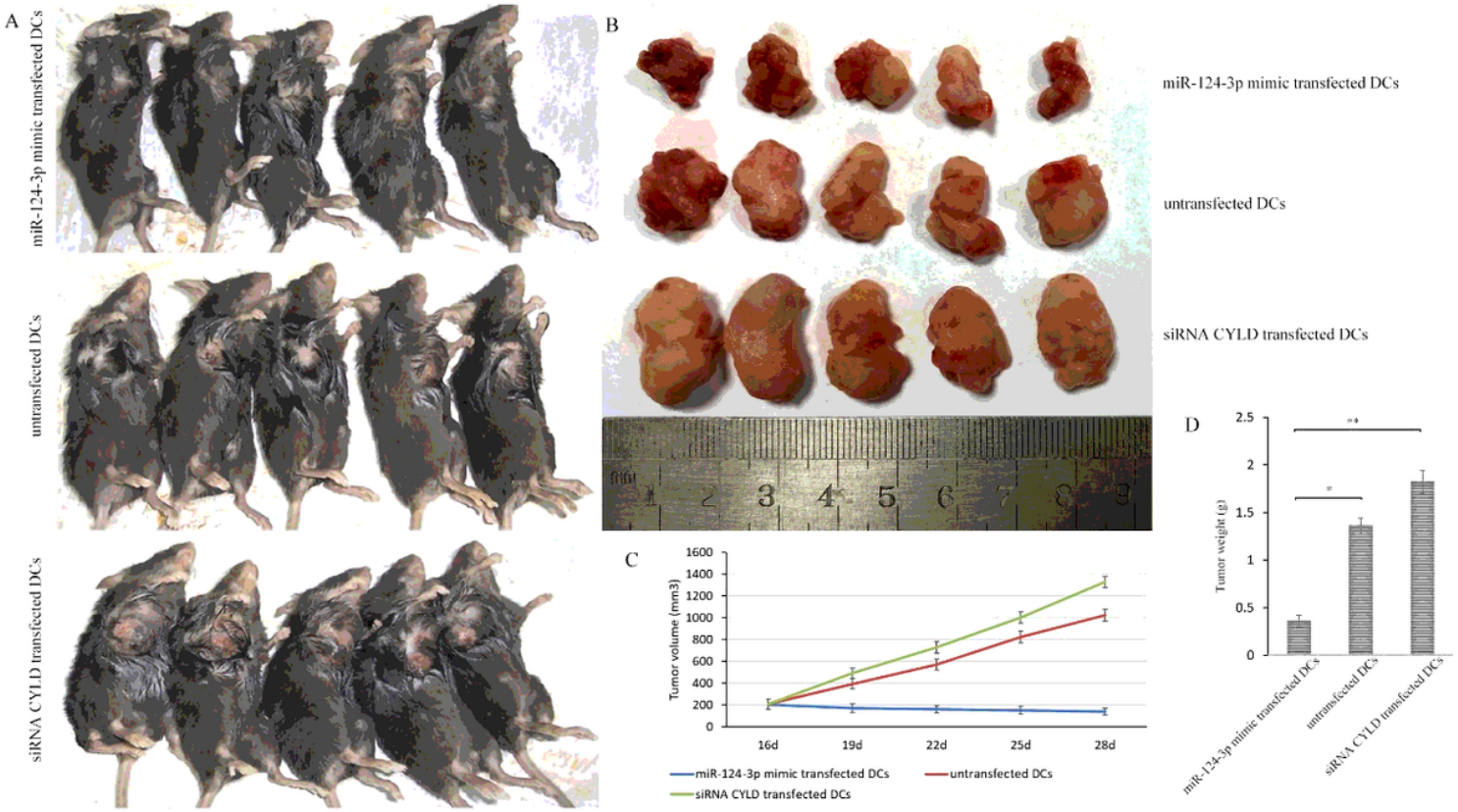

Figure 5

miR-124-3p suppresses the proliferation of lung tumors by targeting CYLD in vivo. C57BL/6 mice were injected with miR-124-3p mimic transfected DCs, siRNA CYLD transfected DCs, or untransfected DCs via the tail vein. (A) C57BL/6 mice were inoculated with LL/2 lung adenocarcinoma cells, allowed to develop tumors for 28 days, and then sacrificed by cervical dislocation. (B) After the mice were sacrificed, tumor tissue was harvested and weighed. Data are shown as mean ± SEM (n=5). *P<0.05; (C) Tumors growth was recorded every 3 days using a caliper, and tumor volume was calculated as a×b2×0.5(a, longest diameter; b, shortest diameter); (D) A tumor growth curve is shown.*P<0.05, **P<0.01.