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LncRNA MALAT1 promotes tumorigenesis and immune escape of diffuse large B cell lymphoma by sponging miR-195

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ABSTRACT

Background: PD-L1 enhanced the tumorigenesis and immune escape abilities of cancers. The upstream mechanisms of PD-L1 in regulating tumorigenesis and immune escape of diffuse large B cell lymphoma (DLBCL) remained unclear.

Methods: Human DLBCL cell line OCI-Ly10 and DLBCL patient samples were used in this study. MALAT1 was knocked down by shRNA. MiR-195 was inhibited by miR-195 inhibitor. Levels of MALAT1, PD-L1, miR-195 and CD8 were detected by RT-qPCR. Protein levels of PD-L1, Ras, p-ERK1/2, ERK1/2, Slug, E-cadherin, N-cadherin, Vimentin were detected by western blotting. The interaction between MALAT1 and miR-195, miR-195 and PD-L1 were detected by luciferase assay. OCI-Ly10 cell proliferation and apoptosis were detected by MTT and Annexin V/PI assays, respectively. Migration was detected by transwell assay. Cytotoxicity of $CD8^+$ T cells was detected by LDH cytotoxicity kit. Proliferation and apoptosis of $CD8^+$ T cell co-cultured with OCI-Ly10 cells were analyzed by CFSE and Annexin V/PI staining.

Results: MALAT1, PD-L1 and CD8 were up-regulated in DLBCL tissues while miR-195 was down-regulated. MiR-195 was negatively correlated with MALAT1 and PD-L1. MALAT1 could sponge miR-195 to regulate the expression of PD-L1. shMALAT1 treatment increased miR-195 level and decreased PD-L1 level. It also inhibited cell proliferation, migration and immune escape ability while increased apoptosis ratio of OCI-Ly10 cells. shMALAT1 treatment in OCI-Ly10 cells also promoted proliferation and inhibited apoptosis of $CD8^+$ T cells. Knocking down of MALAT1 also suppressed EMT-like process via Ras/ERK signaling pathway. These effects were all rescued by miR-195 inhibitor.

Conclusion: Long non-coding RNA MALAT1 sponged miR-195 to regulate proliferation, apoptosis and migration and immune escape abilities of DLBCL by regulation of PD-L1.

1. Introduction

Diffuse large B cell lymphoma (DLBCL) is a common adult non-Hodgkin lymphoma (NHL), a cancer of B cells. DLBCL mostly occurred in aged populations and could also be observed in children in rare cases [1]. The 5-year survival rate range from 60%~70% for patients who received first line therapies, but 50% of these patients would suffer refractory and their median OS (overall survival) was less than 10 months [2]. As a lymphoma, DLBCL could arise in any part of human body and bring great threaten to patients' lives. Although current therapies could extend the life cycle of patients and improve their life quality, the anticancer response and prognosis are still very poor [3–5].

Immune system plays critical role in tumor progression. The microenvironment of DLBCL contains lots of T cells, B cells, dendritic cells (DC) and many cytokines [6]. Although immune system could eliminate invaders in many cases, cancer cells must have developed the ability called “immune escape” to evade immune surveillance [7]. Immune escape refers to the phenomenon by which tumor cells can grow and metastasize by avoiding recognition and attack by the immune system through various mechanisms, which is an important strategy for cancer proliferation, survival and development [8,9]. Re-activating the immune system is the central concept of cancer immune therapy [10,11].

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The immune escape mechanisms in non-Hodgkin lymphoma are not well characterized. However, over-expression of a set of genes including PD-1, PD-L1, PD-L2 and LAG3 were observed in DLBCL cells with immune escape [6]. Study of the role and mechanism of PD-L1 in DLBCL immune escape would help to understand more about DLBCL immunotherapy and may support future studies to improve immunotherapy efficacy.

PD-L1 is a B7 homology family molecule. It is an immune regulatory factor and an important checkpoint in immune response. When interacting with PD-1 and CD80, PD-L1 could inhibit T cell function [10,12]. PD-1 and its ligands B7-H1/PD-L1 and B7-DC/PD-L2 thus played pivotal role in maintaining an immunosuppressive situation in tumor microenvironment. Over-expression of PD-L1 promoted cell proliferation, epithelial-mesenchymal transition (EMT) and facilitated immune escape of breast cancer, lung cancer and many other cancers [10,12–14]. Cancer cells could in turn enhance PD-L1 expression and decrease immune response, enhancing their immune escape ability. PD-1/PD-L1 inhibition therefore was developed as an important therapy method in many cancers [10,12]. A series of immune checkpoint inhibitors targeting PD-1/PD-L1 pathway such as Nivolumab and Pembrolizumab emerged as effective treatments in PD-L1-positive cancers, however, the objective response rate was not satisfying [15,16]. The objective response rate of Nivolumab in DLBCL was only 36% [17]. MiR-195 was considered as cancer suppressor in many cancers including lung cancer, cervical cancer and breast cancer [18–20]. MiR-195 was down-regulated in DLBCL [21], indicating potential connection between miR-195 and DLBCL progression. Through bioinformatics analysis (Targetscan) we also predicted direct interaction between miR-195 and PD-L1, indicating that miR-195 might regulate PD-L1 expression.

Long non-coding RNA (LncRNA) is > 200 nt in length, and participates in a variety of cellular processes including cell migration, invasion, and also cell proliferation in tumors [22]. Although it was once considered as noise RNA, it emerged as key regulatory factors of gene expression in recent studies. LncRNA could affect gene expression via interacting with miRNA, and affect its target genes, presumably by forming ceRNA network [23,24]. It was reported that lncRNA MALAT1 was up-regulated in DLBCL cells [25]. According to reports and bioinformatics prediction, MALAT1 could regulate miR-195 expression in human hepatocellular carcinoma [26], through which it affected cancer proliferation and metastasis [18–21]. Although little is known about the functions and mechanisms of MALAT1 in DLBCL, it's possible that MALAT1 could regulate DLBCL progression through regulating immune escape and tumorigenesis via targeting miR-195/PD-L1 axis.

In this research, we focused on uncovering the upstream mechanisms of PD-L1 regulation and tried to describe the regulatory networks between lncRNA MALAT1, miR-195 and PD-L1 in DLBCL. We elucidated that the long non-coding RNA MALAT1 could affect proliferation, apoptosis, migration and immune escape abilities of DLBCL by regulating miR-195 and PD-L1. We also validated the critical roles of Ras/ERK/EMT signal pathway during this process. We firstly uncovered the important function of MALAT1/miR-195/PD-L1 regulatory network in DLBCL progression. This study would provide support for future researches and may elucidate another way to improve therapeutic approaches of DLBCL immunotherapy.

2. Materials and methods

2.1. Clinical specimens

A total of 37 patients pathologically diagnosed with diffuse large B cell lymphoma in the First and Second Affiliated Hospital of Nanchang University (Nanchang, Jiangxi, China) were recruited into this study. None of the patients received either radiotherapy or chemotherapy prior to tissue harvesting. All samples were immediately frozen and stored in a liquid nitrogen canister at −80 °C until use. The present study was approved by the Ethics Committee of the First and Second Affiliated Hospital of Nanchang University and all patients provided written informed consent.

2.2. Cell culture

Human DLBCL cell line OCI-Ly10 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, US) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, US) and 1% penicillin-streptomycin solution (Gibco, Grand Island, NY, US) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell transfection

MiR-195 mimics, inhibitor and its scrambled control (NC) were synthesized by GenePharma (Shanghai, China) and cloned into pcDNA3.1 vectors (Invitrogen, Carlsbad, CA, USA). The short hairpin RNA (shRNA) targeting MALAT1, and scrambled control shRNA (shNC) were also obtained from GenePharma (Shanghai, China). After being cultured to 70–80% confluence, cells were transfected with indicated miR-195 mimics, miR-195 inhibitor or shMALAT1 using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. All the transfected cells were then cultured for 48 h before different assays.

2.4. Dual luciferase reporter assay

Direct interactions between miR-195 and PD-L1 were predicted by Targetscan website (http://www.targetscan.org/vert_72/). Luciferase reporter assay was performed by co-transfecting firefly luciferase reporter plasmids containing MALAT1 or PD-L1 of their wild type (WT) or mutant (MUT) and renilla luciferase control reporter vectors (Promega, Madison, Wisconsin, USA), and miR-195 mimics or miR-195 NC by lipofectamine 2000 into 293T cells. 48 h after transfection, luciferase assay was conducted using Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instruction. Briefly, cells were lysed and 100 μL supernatant were transferred into luminometer tubes, mixed with 20 μL luciferase assay reagent and signals were detected on a GloMax20/20 luminometer (Promega, Madison, Wisconsin, USA).

2.5. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted for cell viability detection. OCI-Ly10 cells were cultured into 96-well plates with 100 μL of growth medium. After required treatment, cells in the plates were centrifuged, and the supernatants were discarded, followed by rinsed with PBS once and administrated with 20 μL MTT (5 mg/mL) for 4 h at 37 °C. 150 μL DMSO was used to dissolve formazan precipitation. Absorbance at 490 nm was detected using a microplate reader (BioTek, Hercules, CA, USA).

2.6. Transwell assay

OCI-Ly10 cells migration assay was carried out with a chamber containing a polycarbonate membrane (8 μm pore size). After being collected by centrifugation, OCI-Ly10 cells after indicated treatment were re-suspended in serum-free RPMI-1640 medium. Then, 100 μL cell suspension was placed into the upper transwell chamber while medium containing 10% FBS was placed in the lower chamber. 24 h later, the number of cells that migrated to the lower chamber was counted.

2.7. Annexin-V/PI assay

OCI-Ly10 cells or $CD8^+$ T cells were harvested and washed once

with PBS. The cell pellets were then suspended in staining buffer (PBS, 0.01% NaN_3) at a concentration of 1×10^7 cells/mL. 100 μL cell suspension was incubated with Annexin-V-FITC and PI (Sigma-Aldrich, USA) for 15 mins in dark. Cells were then washed with the staining buffer. The samples were subjected to flow cytometric analysis using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The apoptosis of $CD8^+$ T cells was carried out after being co-cultured with OCI-Ly10 cells.

2.8. $CD8^+$ T cell preparation and LDH cytotoxicity assay

$CD8^+$ T cells from peripheral blood mononuclear cells that obtained from healthy donors were isolated with Dynabeads™ CD8 (Invitrogen, Carlsbad, CA, USA) following manufactures' instructions. $CD8^+$ T cells were co-cultured with OCI-Ly10 cells at a ratio of 1:0, 2:1, 3:1, 5:1 and 0:1 as indicated. After 24 h, T cell cytotoxicity was detected by LDH cytotoxicity kit (ThermoFisher, Waltham, MA, USA) following manufacturer's instructions.

$$\text{Cytotoxicity} = [1 - (OD_{case} - OD_{effector\ cell})/OD_{target\ cell}] \times 100\%.$$

2.9. $CD8^+$ T cell proliferation assay

Proliferation of $CD8^+$ T cells was analyzed by carboxyfluorescein succinimidyl ester (CFSE) assay. $CD8^+$ T cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Gibco, Carlsbad, CA) and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (BD Biosciences, San Jose, CA, USA). Cells after indicated treatment were harvested and stained with CD3-APC and CD28-PerCP antibodies and analyzed by flow cytometry. Cells with low CFSE signal were considered as proliferated cells.

2.10. Immunohistochemistry assay

Immunohistochemical staining for CD8 in tumor tissues was performed. Briefly, formalin-fixed and paraffin-embedded samples from patients were cut into sections as thin as 5 μm with a microtome. Sections were incubated with primary anti-CD8 antibody (Abcam, Cambridge, MA) and then incubated with the secondary anti-mouse IgG-biotin antibody. The signal of a target protein was developed using Diaminobenzidine (DAB) substrate and the slides were counterstained with hematoxylin. All stained sections were imaged under a light microscope (Zeiss, Jena, Germany).

2.11. RNA extraction and quantitative real-time PCR

Total RNA was extracted from DLBCL tissue samples and cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at −80 °C. cDNA was synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit (Takara, Dalian, China) and stored at −20 °C. Quantity and quality of nucleic acid were assessed by Nanodrop (ThermoFisher, Waltham, MA, USA). Oligonucleotide primers of lncRNA MALAT1, miR-195, PD-L1 and CD8 were synthesized and quantitative PCR reactions were performed using 2× SYBR Green master mix Kit following manufactures' instructions (ThermoFisher, Waltham, MA, USA) in Applied Biosystems 7500 Real Time PCR System (ThermoFisher, Waltham, MA, USA) with standard cycling mode (95 °C 1 min, and 40 cycles of 95 °C 15 s, 60 °C 15 s, 72 °C 1 min) in a total reaction volume of 10 μL. The gene expression levels for all samples were normalized to U6snRNA (for miRNA) or GAPDH expression using the $2^{-\Delta\Delta Ct}$ method. Experimental and control groups were defined as described in the corresponding figure legends. Samples were prepared in triplicates. The primers sequences in the current study were listed below:

MALAT1-F: 5′-CTGCACATTAGCAATTTCAGCAA-3′;
MALAT1-R: 5′-GGCTCCTTTAGTCCCTTCTGAG-3′.
MiR-195-F: 5′-GGGGAGCCAAAAGGGTCATCATCT-3′;
MiR-195-R: 5′-GAGGGGCCATCCACAGTCTTCT-3′.
PD-L1-F: 5′-TGGCATTTGCTGAACGCATTT-3′;
PD-L1-R: 5′-TGCAGCCAGGTCTAATTGTTTT-3′.
CD8-F: 5′-AAACTCGCATCTACTGGCAAA-3′
CD8-R: 5′-GGTTCTTGTACTCGGGCCATA-3′
U6snRNA-F: 5′-CTCGCTTCGGCAGCACA-3′;
U6snRNA-R: 5′-AACGCTTCACGAATTTGCGT-3′.
GAPDH-F: 5′-TGTGGGCATCAATGGATTTGG-3′;
GAPDH-R: 5′-ACACCATGTATTCCGGGTCAAT-3′.

2.12. Western blot analysis

Cells were harvested and lysed in the RIPA buffer (Sigma-Aldrich, Burlington, Massachusetts, USA). Protein concentrations were determined using the BCA protein assay kit (ThermoFisher, Waltham, MA, USA). 30 μg proteins were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 8.0) containing 5% non-fat milk, the membranes were incubated with indicated primary antibodies overnight at 4 °C. PD-L1, Ras, p-ERK1/2, ERK1/2, Slug, E-cadherin, N-cadherin, Vimentin antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA) and diluted at 1:1000 following manufacturer's instructions. Following extensive washing, the membranes were then incubated with secondary peroxidase-linked goat anti-rabbit IgG for 1 h at room temperature. The immunoreactivity was visualized by enhanced chemiluminescence (ThermoFisher, Waltham, MA, USA). The proteins were quantified using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.13. Statistical analysis

Data were analyzed with Prism 6.0 (GraphPad Software, USA). Each experiment was performed as least three times. The data are shown as the mean ± standard deviation (SD). Spearman correlation analysis was performed to analyzed the correlation between MALAT1, miR-195, PD-L1 and CD8 in DLBCL tissues. Statistical evaluation was performed using Student's *t*-test (two tailed) between two groups or one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparison. $p < 0.05$ was considered significantly different.

3. Results

3.1. MALTA1, miR-195, PD-L1 and CD8 were differently expressed in DLBCL tissues

We first analyzed the levels of MALAT1, miR-195 and PD-L1 by RT-qPCR in DLBCL tissues collected from 37 DLBCL patients, normal lymph node tissues were used as controls. The expressions of MALAT1 and PD-L1 were dramatically higher in tumor tissues. On the contrary, miR-195 was significantly decreased in tumor samples (Fig. 1A–C). To elucidate whether there are correlations between their expression patterns, we conducted Spearman correlation analysis between each two of MALAT1, miR-195 and PD-L1. From the results, we found that MALAT1 was negatively correlated with miR-195 (Fig. 1D) and positively correlated with PD-L1 (Fig. 1F). In addition, PD-L1 was negatively correlated with miR-195 (Fig. 1E). CD8 expression was also detected by RT-qPCR and immunohistochemistry and it was upregulated in tumor samples (Fig. 1G and I). PD-L1 and CD8 were positively correlated with each other as well (Fig. 1H). Thus, expression of MALAT1, miR-195, PD-L1 and CD8 might be correlated with DLBCL progression.

3.2. Knocking down MALAT1 affected cell proliferation, apoptosis, migration and resistance to cytotoxicity of $CD8^+$ T cells

We tried to manipulate MALAT1 level by transfecting shMALAT1 to

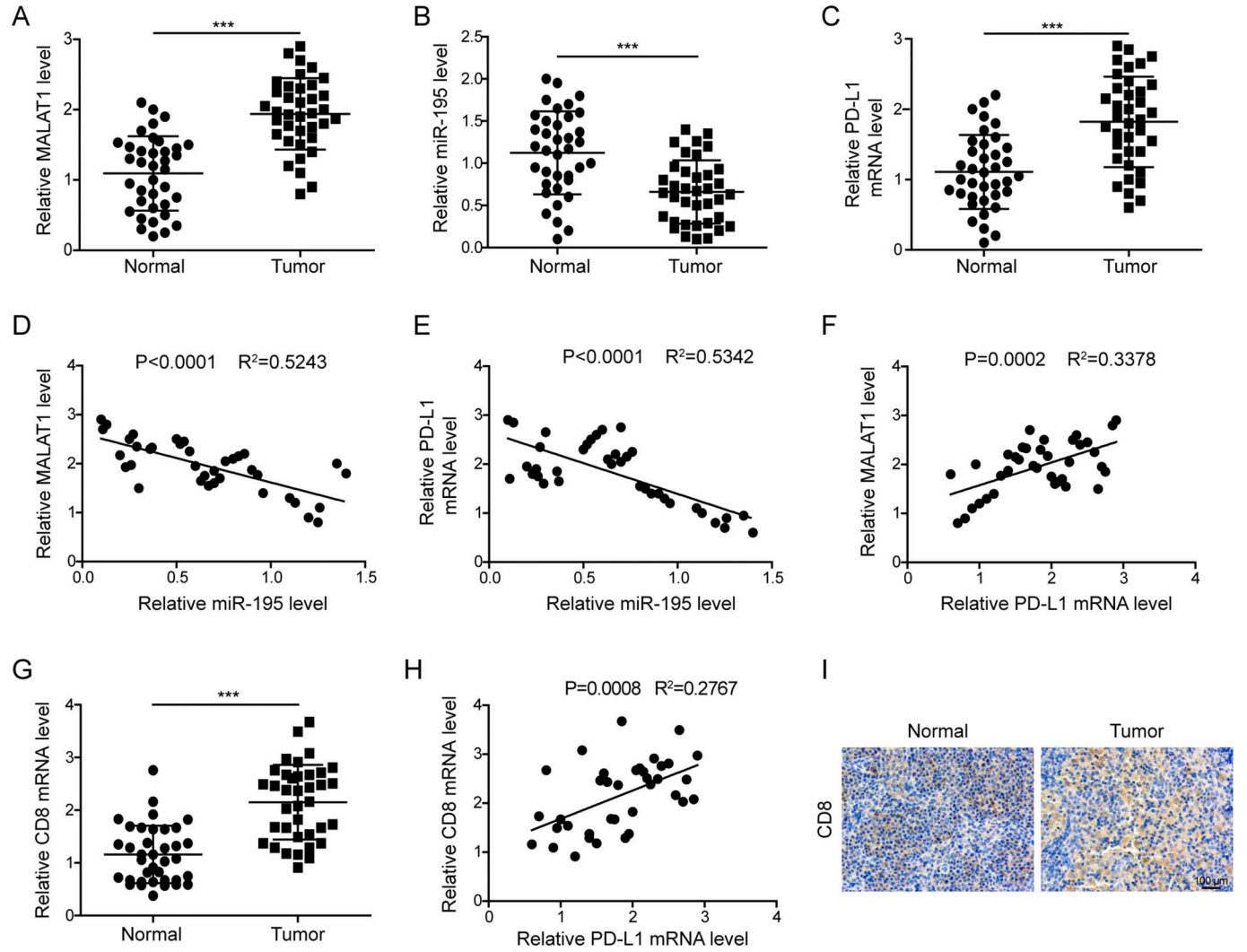

Fig. 1. MALTA1, miR-195, PD-L1 and CD8 were differently expressed in DLBCL tissues. (A–C) DLBCL and normal lymph node tissues were collected and detected for MALTA1, miR-195 and PD-L1 expression by RT-qPCR. N = 37. (D–F) Spearman correlation analysis between each two of MALAT1, miR-195 and PD-L1. (G) DLBCL and normal lymph node tissues were collected and detected for CD8 expression by RT-qPCR. N = 37. (H) Spearman correlation analysis between PD-L1 and CD8. (I) Immunohistochemistry results of CD8 in tumor and normal tissues. ***$p < 0.001$.

study its role on tumor progression of DLBCL. We successfully down-regulated MALAT1 level by transfecting shMALAT1 into DLBCL cell line OCI-Ly10 (Fig. 2A). Cell viability was dramatically decreased by shMALAT1 (Fig. 2B). Meanwhile, apoptosis ratio of these shMALAT1 treated cells was significantly enhanced (Fig. 2C and D). Cell migration was inhibited by shMALAT1 treatment too (Fig. 2E). We next detected EMT-related proteins by western blotting and found that the epithelial marker E-cadherin was promoted in shMALAT1 treated cells, while mesenchymal markers N-cadherin, Vimentin and transcription factor Slug were decreased (Fig. 2F and G). Also, cytotoxicity of $CD8^+$ T cells to OCI-Ly10 cells was promoted by shMALAT1 treatment and was enhanced with higher ratio of $CD8^+$ T cells (Fig. 2H). We therefore concluded that MALAT1 played pivotal roles in promoting DLBCL proliferation, migration, EMT-like process and also enhancing resistance to cytotoxicity of $CD8^+$ T cells.

3.3. MALAT1 directly sponged miR-195 to regulate PD-L1 expression

In DLBCL cells, shMALAT1 treatment significantly increased miR-195 level while decreased PD-L1 mRNA level (Fig. 3A and B). The corresponding protein level of PD-L1 exerted similar pattern (Fig. 3C and D). We thus analyzed the sequence of MALAT1, PD-L1 and miR-195. As shown in Fig. 3E and F, we found highly homologous complementary region between MALAT1 and miR-195, and between miR-195 and PD-L1 3′UTR. The luciferase activity was significantly down-regulated in the MALAT1-WT group, while no effect was observed in the MUT group when the miR-195 was over-expressed (Fig. 3G). In addition, miR-195 mimics significantly reduced the wide type of PD-L1 3′UTR-driven luciferase activity (Fig. 3H). These provided evidence for a direct interaction and regulation between MALAT1 and miR-195, and between miR-195 and PD-L1. Taken together, MALAT1 might induce the expression of PD-L1 to modulate cell proliferation, apoptosis, migration and resistance to cytotoxicity of $CD8^+$ T cells by sponging miR-195.

3.4. MiR-195 inhibitor reversed the effects on proliferation, apoptosis and migration and sensitivity to cytotoxicity of $CD8^+$ T cells induced by shMALAT1

To find more evidence for our notion that MALAT1 regulated DLBCL progression through regulating miR-195, we co-transfected shMALAT1 and miR-195 inhibitor in DLBCL cells. MALAT1 knocking down increased expression of miR-195, and this effect was reversed by administration of miR-195 inhibitor (Fig. 4A). As for expression of PD-L1,

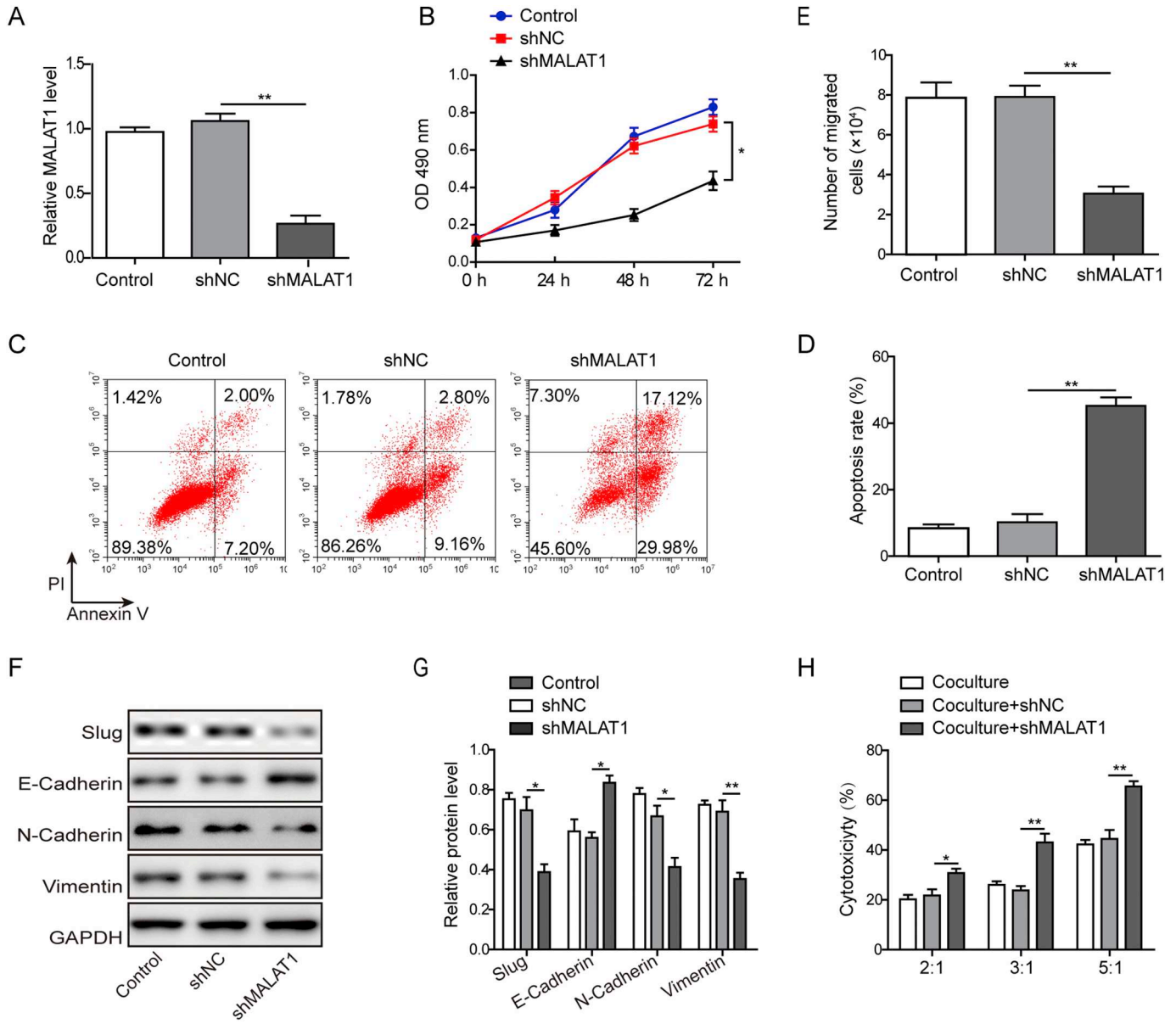

Fig. 2. Knocking down MALAT1 affected cell proliferation, apoptosis, migration and resistance to cytotoxicity of $CD8^+$ T cells. (A) Relative MALAT1 level was detected by RT-qPCR after shMALAT1 treatment. (B) Cell proliferation was detected by MTT assay for 24, 48 and 72 h. (C–D) Cell apoptosis was detected by Annexin V/PI assay. (E) Cell migration ability was detected by transwell assay. (F–G) EMT markers Slug, E-cadherin, N-cadherin and Vimentin were detected by western blotting. (H) Cytotoxicity of $CD8^+$ T cells was detected by LDH cytotoxicity kit. $^{**}p < 0.01$, and $^{*}p < 0.05$.

knocking down of MALAT1 decreased mRNA level of PD-L1, and miR-195 inhibitor restored PD-L1 mRNA level to the normal level (Fig. 4B). This result was further confirmed by western blotting, PD-L1 protein level was dramatically suppressed in shMALAL1 treated group, and was restored by additional miR-195 inhibitor (Fig. 4C and D). We also detected other biological processes. Cell viability was decreased by shMALAT1 and restored by miR-195 inhibitor (Fig. 4E). Migration of DLBCL cells was suppressed by shMALAL1, and such effect was reversed by miR-195 inhibitor too (Fig. 4H). MALAT1 knocking down increased apoptotic DLBCL cells and additional miR-195 inhibitor partly restored the apoptosis ratio (Fig. 4F and G). To find out whether MALAT1 could affect immune escape by sponging miR-195, we decided to detect susceptibility of DLBCL cells to $CD8^+$ T cells. Cytotoxicity of $CD8^+$ T cells could reflect DLBCL susceptibility and was detected by LDH cytotoxicity kit. In cells with shMALAT1 treatment, cytotoxicity was dramatically increased comparing to control group (Fig. 4I). Additional miR-195 inhibitor decreased cytotoxicity while miR-195 NC exerted no such effect (Fig. 4I). These results supported our theory that MALAT1 could regulate miR-195 and then affect DLBCL progression.

3.5. MiR-195 inhibitor reversed the effects of shMALAT1 on EMT-like process via Ras/ERK signaling pathway

Since we have deduced a regulatory network among MALAT1, miR-195 and PD-L1, we next introduced miR-195 inhibitor into cells with shMALAT1 to confirm this finding by detecting EMT markers. Slug, N-cadherin, Vimentin were all down-regulated by knocking down of MALAT1, while E-cadherin was up-regulated under the same condition, representing the decreased EMT-like process was caused by shMALAT1 (Fig. 5A and B). But, miR-195 inhibitor rescued the expression of Slug, N-cadherin, Vimentin and E-cadherin induced by shMALAT1 (Fig. 5A and B). Because some reports demonstrated that PD-L1 conferred malignancy via Ras/ERK/EMT activation [27], the levels of Ras, total ERK1/2 and p-ERK1/2 were then studied. Our results showed that

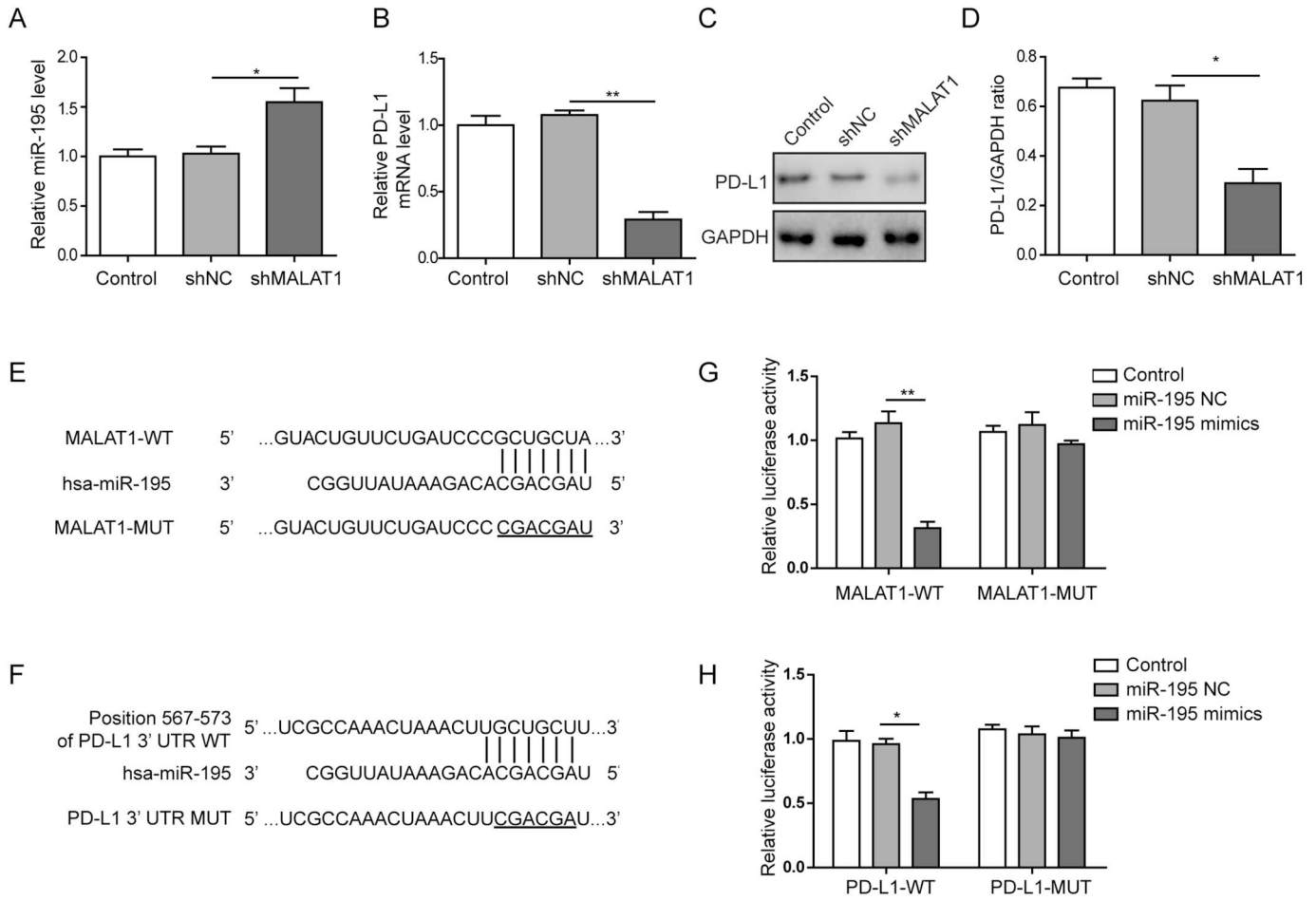

Fig. 3. MALAT1 directly sponged miR-195 to regulate PD-L1 expression. (A) MiR-195 level was detected by RT-qPCR after shMALAT1 treatment. (B) PD-L1 mRNA level was detected by RT-qPCR after shMALAT1 treatment. (C–D) PD-L1 protein level was detected by western blotting and GAPDH served as loading control. (E) Bioinformatics analysis of interaction between MALAT1 and miR-195. (F) Bioinformatics analysis of interaction between PD-L1 and miR-195. (G) The relative luciferase activity in MALAT1-WT and MALAT1-MUT after transfected with miR-195 mimics or NC. (H) The relative luciferase activity in PD-L1-WT and PD-L1-MUT after transfected with miR-195 mimics or NC. The luciferase reporters containing the predicted binding sties of lncRNA MALAT1 and PD-L1 3′UTR were co-transfected with miR-195 mimics or NC, and then the luciferase activity was measured after 48 h. $^{**}p < 0.01$, and $^{*}p < 0.05$.

shMALAT1 treatment caused the decreased Ras and p-ERK1/2, but miR-195 inhibitor rescued such effect (Fig. 5A and B). These indicated that MALAT1 inhibited miR-195 to enhance PD-L1 expression and then promoted EMT-like process, possibly due to activation of Ras/ERK signaling pathway.

3.6. MALAT1 in DLBCL cells regulated proliferation and apoptosis of CD8$^+$ T cells by targeting miR-195

CD8$^+$ T cells were co-cultured with OCI-Ly10 cells with shMALAT1 and miR-195 inhibitor treatment. Proliferation of CD8$^+$ T cells was detected by CFSE staining assay. Cell proliferation was significantly inhibited after co-culturing with OCI-Ly10 cells. Then, shMALAT1 treatment in OCI-Ly10 cells increased cell proliferation of CD8$^+$ T cells. MiR-195 inhibitor reversed the effects induced by shMALAT1 treatment (Fig. 6A and B). Apoptosis of CD8$^+$ T cells was also detected. In non-cocultured groups, little apoptosis was observed. But, apoptosis of CD8$^+$ T cells was increased dramatically in co-cultured group. shMALAT1 treatment in OCI-Ly10 cells partially inhibited the apoptosis of CD8$^+$ T cells. Additional miR-195 inhibitor rescued the effects on apoptosis of CD8$^+$ T cells induced by shMALAT1 treatment (Fig. 6C and D). These results showed that MALAT1 in DLBCL cells suppressed proliferation and promoted apoptosis of CD8$^+$ T cells by targeting miR-195.

4. Discussion

One of the most important hallmark of cancer cells is that they could evade supervision from immune system, this is called immune escape [6,7,21]. Cancer cells cheated immune system or blocked immune cells through mysterious mechanisms. PD-1/PD-L1 emerged in recent studies of immune escape and shed light on that mystery like a rising star. Inhibition of this pathway exerted remarkable therapeutic responses in many cancers including melanoma, lung cancer, glioblastoma and many other cancers [13,28–30]. Cells with high PD-L1 expression inhibited CD8$^+$ T cell expansion and cytotoxic ability [14,31]. PD-L1 positive cells therefore developed into much serious tumor progression. In our study, over-expressed PD-L1 was found in DLBCL tissues, indicating PD-L1-mediated immune escape might contribute to DLBCL tumorigenesis. Studying the upstream regulatory mechanisms of PD-L1 in DLBCL would provide theoretical support and may elucidate other way to improve therapeutic approaches to DLBCL.

MiR-195 was reported as a regulator of PD-L1 [21], we firstly found that miR-195 was decreased and PD-L1 was enhanced in DLBCL tissues with a negative correlation. In addition, we further found miR-195 significantly reduced the wide type of PD-L1 3′UTR-driven luciferase activity. MALAT1 was enhanced in DLBCL tissues, a negative correlation between MALAT1 and miR-195 was also in consistent with previous report that MALAT1 could regulate miR-195 in human hepatoma [26]. Given the positive correlation between MALAT1 and PD-L1, and

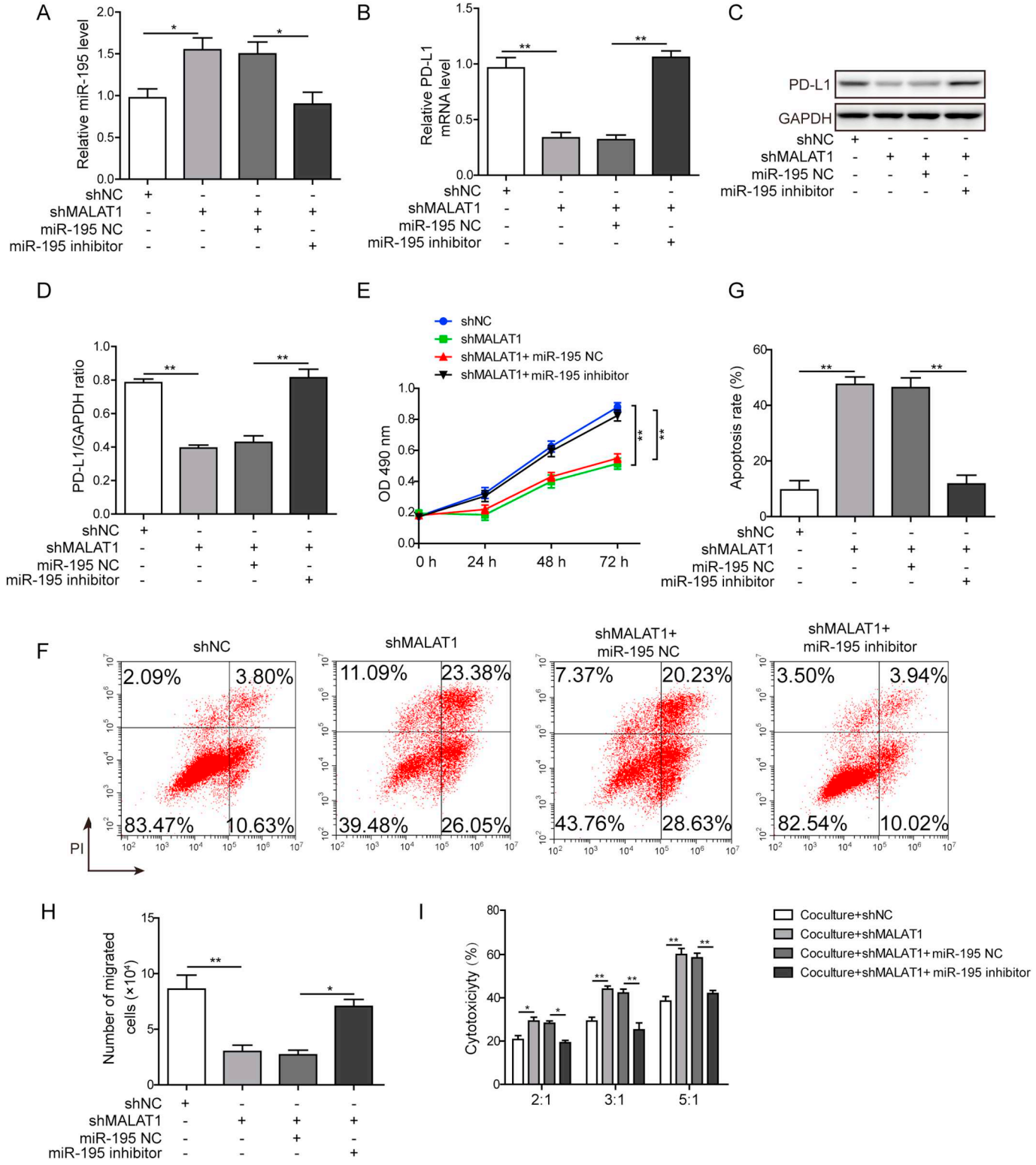

Fig. 4. MiR-195 inhibitor reversed the effects on proliferation, apoptosis and migration and sensitivity to cytotoxicity of $CD8^+$ T cells induced by shMALAT1. (A) MiR-195 level was detected by RT-qPCR after shMALAT1 and miR-195 inhibitor treatment. (B) PD-L1 mRNA level was detected by RT-qPCR after shMALAT1 and miR-195 inhibitor treatment. (C–D) PD-L1 protein level was detected by western blotting and GAPDH served as loading control. (E) Cell proliferation was detected by MTT assay for 24, 48 and 72 h after shMALAT1 and miR-195 inhibitor treatment. (F–G) Cell apoptosis was detected by Annexin V/PI assay. (H) Cell migration ability was detected by transwell assay. (I) Cytotoxicity of $CD8^+$ T cells was detected by LDH cytotoxicity kit. $^{**}p < 0.01$, and $^{*}p < 0.05$.

our bioinformatics analysis, together with the decreased luciferase signal in cells co-transfected with MALAT1-WT reporter plasmids and miR-195 mimics, we reasoned that MALAT1 negatively regulated miR-195, and then upregulated its target gene PD-L1, possibly by forming a ceRNA network. This is the first study addressing the MALAT1/miR-195/PD-L1 axis in DLBCL.

MALAT1 was identified as key regulator of miR-195 in many other diseases such as renal carcinoma, osteosarcoma and prostate cancer

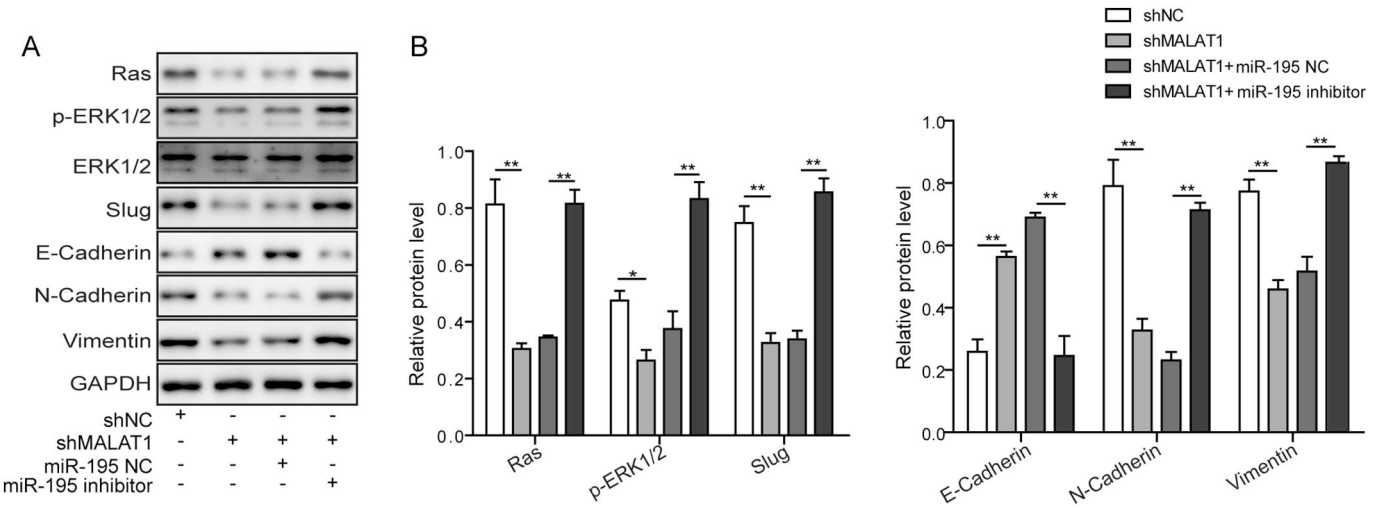

Fig. 5. MiR-195 inhibitor reversed the effects of shMALAT1 on EMT-like process via Ras/ERK signaling pathway. (A) EMT markers Slug, E-cadherin, N-cadherin and Vimentin and Ras, ERK1/2 and p-ERK1/2 were detected by western blotting, GAPDH served as loading control. (B) Statistic analysis in (A). $^{**}p < 0.01$, and $^{*}p < 0.05$.

[32–34]. We tried to evaluate whether MALAT1 could regulate miR-195 and then affect DLBCL tumorigenesis. Knocking down MALAT1 enhanced miR-195 and decreased PD-L1 expression. PD-L1 over-expression could promote cell proliferation, invasion and EMT process [13,14]. Taken together, in DLBCL cells, we speculated that MALAT1 might directly interact with miR-195 and suppress its expression, which promoted PD-L1 expression and DLBCL tumorigenesis. We firstly explained their ceRNA regulatory network in DLBCL cells. Then, we knocked down MALAT1 to investigate its functions in DLBCL. Knocking down MALAT1 decreased cell proliferation, migration and promoted apoptosis. We also found that cytotoxicity of $CD8^+$ T cells to OCI-Ly10 cells was promoted by shMALAT1 treatment and was enhanced with higher ratio of $CD8^+$ T cells. These results demonstrated that MALAT1 promoted DLBCL progression via promoting proliferation, migration, and inhibiting its susceptibility to $CD8^+$ T cells.

For further validation of our findings, we co-transfected miR-195 inhibitor into shMALAT1 treated DLBCL cells. Effects on cell proliferation, migration, apoptosis and cytotoxicity of $CD8^+$ T cells induced by shMALAT1 were all reversed by miR-195 inhibitor. The expression of miR-195 and PD-L1 were also reversed by miR-195 inhibitor. Because miR-195 was reported to be associated with these processes [20,35,36], and MALAT1 targeted miR-195, we deduced that MALAT1 regulated all these processes through directly inhibiting miR-195.

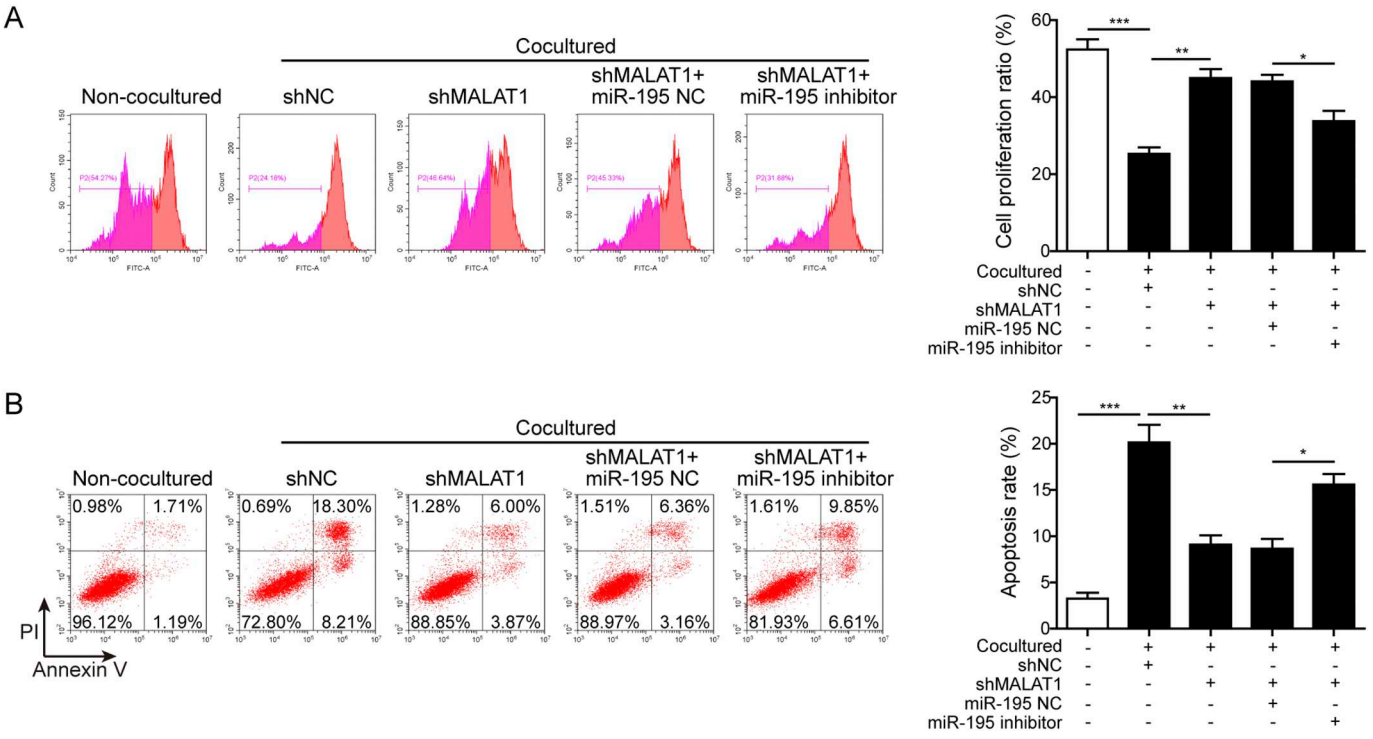

Fig. 6. MALAT1 in DLBCL cells regulated proliferation and apoptosis of $CD8^+$ T cells by targeting miR-195. (A) Proliferation of $CD8^+$ T cells was detected by CFSE staining assay. (B) Apoptosis of $CD8^+$ T cells was detected by Annexin V/PI assay. $CD8^+$ T cells were co-cultured with OCI-Ly10 cells with shMALAT1 and miR-195 inhibitor treatment. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

To find more evidence supporting the theory that MALAT1 could regulate miR-195/PD-L1 axis, we detected EMT markers of DLBCL cells, because previous reports showed that PD-L1 could facilitate EMT process in lung cancer [13]. E-cadherin is usually considered as an epithelial marker, while N-cadherin and Vimentin are mesenchymal markers. What's more, some studies showed EMT- and MET-related processes were important for disease progression, prognosis, and therapeutic opportunities in nonepithelial tumors [37,38]. shMALAT1 treatment decreased the expression of N-cadherin and Vimentin while elevated E-cadherin, meaning that MALAT1 could promote EMT-like process. Additional miR-195 inhibitor reversed the effects of shMALAT1 treatment on EMT markers. We also analyzed the mechanisms behind MALAT1/miR-195/PD-L1 axis. MALAT1 promoted EMT-like process by activating Ras/ERK signal pathway. ERK activation could be reflected by enhanced p-ERK1/2 ratio [39]. Decreased Ras, p-ERK1/2 levels induced by shMALAT1 were rescued by additional miR-195 inhibitor. Thus, MALAT1 could promote EMT-like process by activating Ras/ERK signal pathway through miR-195/PD-L1 axis.

One of the major reasons of PD-1/PD-L1 system-induced immune escape involves cell anergy and cell apoptosis of $CD8^+$ T cells. In our study, proliferation and apoptosis of $CD8^+$ T cells were respectively inhibited and induced after co-culturing with OCI-Ly10 cells. shMALAT1 treatment in OCI-Ly10 cells increased cell proliferation and suppressing cell apoptosis of $CD8^+$ T cells. MiR-195 inhibitor reversed the above effects induced by shMALAT1 treatment. Therefore, our results showed that MALAT1 in DLBCL cells suppressed proliferation and promoted apoptosis of $CD8^+$ T cells by targeting miR-195. In addition, CD8 expression was upregulated in tumor samples. So, MALAT1 might regulate cell anergy and cell apoptosis of $CD8^+$ T cells to modulate immune escape in DLBCL.

Our findings firstly revealed a regulatory network between MALAT1, miR-195 and PD-L1 in DLBCL cells in the world. We demonstrated that the long non-coding RNA MALAT1 sponged miR-195 to regulate proliferation, apoptosis and migration and immune escape abilities of DLBCL by regulation of PD-L1. All these biological processes could facilitate cancer progression. Rational therapeutic methods combined the modulation of this axis (for example, directly modulating MALAT1 or miR-195 using mimics or inhibitor) may help to improve efficacy of DLBCL immunotherapy. This finding would provide support for further studies about DLBCL treatment and may bear great therapeutic potential. It is noteworthy that the idea of microRNA sponge or lncRNA sponge is still being debated and there may be other theories explaining the MALAT1/miR-195/PD-L1 axis. Hence, more studies are needed for validation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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