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RESEARCH

Knockdown NEK7 stimulates anti-tumor immune responses by NLRP3/PD-L1 signaling in esophageal cancer

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Abstract

Background Esophageal cancer is a prevalent malignancy with limited treatment options. The study aimed to understand the role and mechanism of NEK7 in esophageal cancer development.

Methods RNA sequencing compared esophageal cancer tissues with adjacent tissues, and real-time PCR validated NEK7 expression. Co-IP identified NLRP3 as NEK7's binding partner. We also study the effects of NEK7 knockdown on cell viability, apoptosis, migration, invasion, and the expression of NLRP3/PD-L1 in esophageal carcinoma cell lines. TIMER 2.0 analyzed immune infiltration. An animal model was used to investigate the impact of NEK7 knockdown on tumor size, survival rates, and immune cell infiltration. Licochalcone B blocked NEK7/NLRP3, enhancing CD8 T cell-mediated tumor killing. PD-1's role in T cell viability was also assessed.

Results NEK7 was observed to be markedly elevated in both tumor tissues of esophageal cancer and EC109 cells. Moreover, silencing NEK7 reduced cell viability, migration, and invasion, while enhancing cell apoptosis in vitro. Knockdown of NEK7 caused a notable reduction in levels of NLRP3 and PD-L1 in EC109 cells. NEK7 expression showed a positive correlation with immune cell infiltration. Knockdown of NEK7 decreased PD-L1 expression, while upregulation of NEK7 increased PD-L1 expression, then reversed by NLRP3 knockdown. In animal studies, NEK7 knockdown reduced tumor size and volume while improving survival. It also promoted CD4 and CD8 T cell infiltration while inhibiting Treg cells and PD-1 + CD4 and CD8 T cells. Licochalcone B blocked NEK7/NLRP3 binding, decreased cell viability of EC109 cells, and enhanced the activity of co-cultured CD8 T cells. Furthermore, Licochalcone B and anti-PD-1 treatment increased the killing ratio of EC109 cells.

Conclusion In conclusion, NEK7 is a key regulator in the progression of esophageal cancer and the immune evasion. Targeting the NEK7/NLRP3 pathway may have therapeutic potential for the treatment of esophageal cancer.

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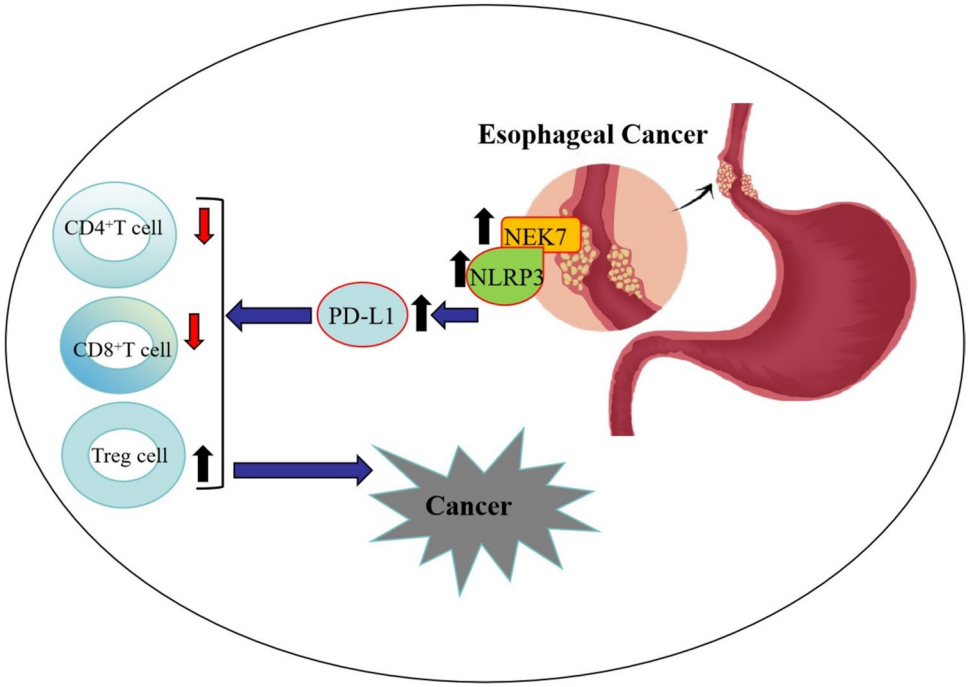
Graphical abstract



Keywords NEK7 · Immune evasion · Esophageal cancer · NEK7/NLRP3 signaling

Introduction

Esophageal cancer is a prevalent malignancy that originates in the esophagus, a muscular tube responsible for transporting food from the throat to the stomach [1]. Squamous cell carcinoma is mostly caused by smoking and drinking [2]. Esophageal cancer symptoms may involve trouble swallowing, pain or discomfort in the chest, unintended weight loss, indigestion, as well as coughing or a hoarse voice [3]. According to the American Cancer Society, esophageal cancer is relatively uncommon in the USA, accounting for about 1% of all cancers diagnosed [4]. But the incidence and fatality rate are high. The incidence of esophageal cancer is increasing in China [5]. Treatment options consist of surgery, radiation therapy, chemotherapy, which are commonly used in the intermediate to advanced stages of the disease. However, there is a growing need for more effective treatments, especially for early-stage esophageal cancer [6]. Therefore, it is crucial to urgently identify new therapeutic targets for advanced esophageal squamous cell carcinoma (ESCC).

Gene therapy is a promising approach for treating cancer, including esophageal cancer. One aspect of gene therapy that researchers are focusing on is targeting the tumor microenvironment [7]. The low activity of NEK7 under natural growth conditions may be important for maintaining homeostasis [8]. However, any disruption of homeostasis may be accompanied by the imbalance of NEK7, leading to abnormal cell growth [9]. In the context of the tumor microenvironment, NEK7 might play a key role in regulating the interactions between cancer cells and their surrounding environment [10]. It has been proposed that NEK7 contributes to tumor development and the spread of cancer cells [11]. Previous research has indicated that NEK7 upregulation is significant in esophageal cancer [12]. Targeting NEK7 in the tumor microenvironment could potentially have therapeutic implications. Therefore, it is crucial to study the function of NEK7 in the therapy of esophageal cancer.

NLRP3 is a gene that encodes a protein playing a crucial role in regulating inflammation and immune responses, particularly through the activation of inflammasomes, which are multi-protein complexes involved in the initiation of inflammatory responses [13]. This gene has been linked to a variety of inflammatory diseases and has been shown to influence the progression of various cancers, including esophageal cancer. Recent studies suggest that galangin, a flavonoid compound, may enhance the anti-tumor activity of esophageal cancer by downregulating NLRP3 inflammasomes [14]. By inhibiting NLRP3, galangin potentially reduces the chronic inflammatory environment

that promotes tumor growth and metastasis, thus improving the overall prognosis for patients suffering from esophageal cancer. On the other hand, PD-L1 (Programmed Death-Ligand 1) is a gene encoding a protein involved in immune regulation, specifically in immune checkpoint pathways [15]. PD-L1 interacts with PD-1, a receptor found on T cells, to suppress the immune response, enabling cancer cells to evade immune detection. Consequently, PD-L1 inhibitors have become a vital approach in cancer immunotherapy. These agents function by disrupting the PD-1/PD-L1 interaction, thus boosting the immune system's ability to target and attack tumor cells. In esophageal cancer, PD-L1 inhibitors are regarded as a primary treatment option for patients in advanced stages, demonstrating considerable promise in increasing survival rates [16]. However, despite the promising applications, research into the regulatory relationship between NEK7, NLRP3, and PD-L1 remains limited, and the exact mechanisms through which NEK7 modulates these pathways in the context of esophageal cancer are still not well understood.

Here, we aim to explore the function and mechanism of NEK7 in the progression of esophageal cancer and support the identification of novel treatment strategies.

Material and methods

Human specimens

Tissue specimens from both esophageal cancer and surrounding non-cancerous tissues were collected from four individuals diagnosed with esophageal cancer. The ages of the patients varied between 23 and 43 years, with an average age of 31.12 ± 1.07 years. Our study received approval from the Medical Ethics Committee of Beijing Luhe Hospital, Capital Medical University, and was conducted in compliance with the Helsinki Declaration. All participants provided informed consent prior to their involvement in the experiment.

Cell culture and treatment

The HET-1A, TE-1, Eca109, and EC109 cell lines were obtained from ATCC. HET-1A cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with EGF and 10% fetal bovine serum. mEC25 cells were maintained in DMEM medium (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS). All cell cultures were kept in a humidified incubator at 37 °C with 5% CO2.

EC109 cells were transfected with sh-NEK7, sh-NLRP-3 with or without pc-NEK7 (GenePharma, Shanghai, China) utilizing Lipofectamine 2000 (Thermo Fisher Scientific, USA). mEC25 cells were transfected with sh-NEK7. After 24 h, the transfected cells were prepared for experimentation. EC109 cells were exposed to 10 μM Licochalcone B for 24 h. After a 24 h treatment, the cells were collected for functional evaluations, and RNA and protein were extracted for Real-time PCR and Western blot analyses, respectively.

Cell proliferation

Cell growth was evaluated through a colorimetric MTT test. Each well of a 96-well plate was seeded with 5,000 cells in 100 μl of DMEM and allowed to settle overnight. After the treatments, the cells were subjected to 500 μg/ml of MTT and kept at 37 °C for 4 h. The medium was then discarded, and the formazan crystals were dissolved in DMSO. The absorbance of the MTT product was recorded at 550 nm using a microplate reader (BioTek Instruments, USA).

Scratch test

Cells with different treatments were cultured in a 6-well plate. Next, a 200-μL yellow pipette tip was used to create horizontal and vertical markings at the bottom of the 6-well plate. Care was taken to maintain uniform intensity to ensure consistent scratch width. The medium was then aspirated, and the plate was gently washed with PBS to remove any cell debris from the scratched area. Serum-free medium was added back, and the plate was observed under a microscope at the appropriate magnification to capture images. Set multiple time points, such as 0 h, 24 h, and repeated the above operations, respectively, and then performed data analysis and statistics.

Transwell experiment

The membrane of the upper Transwell chamber was covered with Matrigel and serum-free medium, then dried in an incubator set to 37 °C for 2 h. Subsequently, a 500-μL serum-free cell suspension, containing 4×10^4 cells, was added to the upper chamber. The lower chamber was filled with 800 μL of medium supplemented with 20% FBS. The assembly was then incubated for 24 h. Following incubation, the cells on the membrane were fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed cells were stained with crystal violet for 15 min. Images of the stained cells were captured using an inverted microscope (Olympus, 400× magnification). The number of migrated cells was determined using ImageJ software.

Flow cytometry

Cell apoptosis was analyzed using the Annexin V-FITC/PI apoptosis analysis kit (YEASEN, cat#40302ES20). One million cells were harvested and incubated with Annexin

V or PI in the dark for 15 min. T cells from fresh mouse tissues were stained with CD4, CD8, and Treg surface markers using CD4-FITC (Clone H129.19, Cat#130,308), CD8-FITC (Clone 53–6.7, Cat#100,705), PD-1-PE/Cy7 (Clone RMP1-30, Cat#109,109), and CD25-PE (Clone 3C7, Cat#101,903) antibodies (BioLegend). Granzyme B-FITC (Clone QA16A02, Cat#372,205, BioLegend) and Perforin-PE (Clone S16009A, Cat#154,305, BioLegend) were used for intracellular staining. FACS Canto II flow cytometer (BD Biosciences) was used to conduct flow cytometry analysis, and the resulting data were analyzed using FlowJo v10 software (BD Biosciences).

Immune infiltration analysis

Using the "GENE" module of TIMER (Tumor Immune Estimation Resource), we examined the relationship between specific genes and the immune cell landscape in esophageal cancer. This analysis offered important perspectives on the possible involvement of immune cells in tumor development and the immune response. The findings from this study deepen our comprehension of the tumor microenvironment and its potential impact on the prognosis and treatment of esophageal cancer.

Co-immunoprecipitation (Co-IP)

EC109 cells were harvested and disrupted with an IP lysis buffer supplemented with PMSF (a protease inhibitor, ST505, Beyotime, China). The entire process was conducted on ice to ensure a cold environment. The supernatants obtained were then incubated overnight at 4 °C with agarose-bound anti-NEK7 antibody (ab195470, Abcam, UK) or IgG (Cat# AC005, ABclonal, Shanghai, China). After incubation, the beads were washed three times, treated with 1X loading buffer (P0015A, Beyotime, China), and heated at 99 °C for 5 min. The beads were then removed, and the samples were analyzed by Western blot and probed with anti-NEK7 (ab195470, Abcam, UK), anti-NLRP3 (ab214185, Abcam, UK), or anti-GAPDH antibodies (Cat# 60,004–1-Ig, RRID: AB_2107436, Proteintech, Wuhan, China).

Mice model and in vivo treatments

C57 mice were sourced from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (CAS). To establish the xenograft mouse model, C57 mice were used for in vivo experimentation. mEC25 cells were first transfected with sh-NEK7 to knock down the expression of NEK7. These transfected mEC25 cells were then harvested and suspended in a suitable medium for injection. Around 1×10^6 cells were injected into the flank of each C57 mouse. The mice were observed regularly for tumor development, and tumor size was measured with calipers. After a set period, the tumors were excised for further analysis. This xenograft model allows for the evaluation of NEK7's role in tumor growth and progression, providing a valuable platform for studying potential therapeutic strategies targeting NEK7 in cancer research.

Killing effect of T cells on tumor cells in vivo

EC109 and T lymphocytes were mixed inoculated in 96-well plate at 1:20. Each hole contains 2×10^4 and 2×10^5 independent T lymphocytes. The antibody against YG-003D3 (Abcam, UK) was diluted to an appropriate concentration with MEM (starting at 400 nm, 5 times, 3 gradients, 5 times). Diluted antibodies were added to the 96-well plate. After 37 °C culture for 12 and 24 h, the supernatant was taken and the anti-tumor effect of T lymphocytes was determined by anti-tumor test in vitro.

Transcriptome sequencing

RNA was isolated using TRIzol® reagent (Invitrogen), as per the product instructions (Invitrogen). The RNA quality was assessed with the 2100 Bioanalyzer (Agilent) and its concentration was determined using the ND-2000 spectrophotometer (NanoDrop Technologies). On this basis, a high-throughput sequencing platform was established by using high-quality nucleic acid samples (OD260/280 = 1.8 ~ 2.2, OD260/230, RIN > 6.5, 28S:18S ≥ 1.0, > 1 μg).

The TruSeqTMRNA samples obtained by Illumina (San Diego, CA) were used to construct a high-throughput RNA-seq platform. To put it simply, RNA was separated from DT microspheres by pola screening method, and then broken up by fragmentation. On this basis, RNA fragments with high specificity were screened by bioinformatics technology. On this basis, genetic engineering was realized by means of Illumina high-throughput sequencing, terminal repair, phosphorylation, a-base addition and so on. The library was scanned with 2% ultra-high molecular weight DNA electrophoresis and amplified 15 times by NEB method. On this basis, the transcriptional group was deeply sequenced by using Illumina HiSeq xten/NovaSeq 6000 sequencers.

GO analysis

Gene Ontology (GO) analysis of DEGs in tumor tissues versus normal tissues was conducted using the DAVID database. A false discovery rate (FDR) threshold of < 0.05 was used to assess statistical significance for enrichment. The resulting enriched GO terms were visualized with the Goplot R package.

KEGG signaling pathway enrichment analysis

Gene expression differences were analyzed using the DESeq2 package in R. Genes exhibiting an adjusted p value < 0.05 and a fold change > 2 were classified as differentially expressed. The KEGG database was subsequently utilized to assess the enrichment of these DEGs in tumor tissues relative to normal tissues. Signaling pathways with a false discovery rate (FDR) below 0.05 were regarded as significantly enriched. The outcomes of the enrichment analysis were then visualized with the GOplot R package.

qRT-PCR

RNA was extracted from tissues or cells using Trizol reagent (Beyotime, Beijing, China), following the manufacturer's protocol. The obtained RNA was used to synthesize cDNA (Beyotime, Beijing, China). PCR reactions were performed using the ABI 7900 fluorescence quantitative PCR system (ABI, USA). The primers used were as follows: NEK7: 5′-ATGGAGGAGCCCGAGAAGAG-3′ and 5′- CTCCTGCTCCAGCTTCTTCC-3′, GAPDH: 5′- GTCTCCTATGACTTCAACAGCG-3′ and 5′- ACCACCCTGTTGGTGTAGCCAA-3′. GAPDH expression served as an internal reference to normalize mRNA levels, and relative quantification was determined using the $2^{-\Delta\Delta CT}$ method.

Western blot

The isolated protein was transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk. An antimicrobial solution was then applied to the membranes and incubated overnight at 4 °C. Next, the membranes were incubated with primary antibodies against NLRP3 (1:2000), NEK7 (1:2000), PD-L1 (1:2000), GAPDH (1:2000), and β-actin (1:2000) for human or mouse samples. These specific antibodies for NLRP3, NEK7, PD-L1, GAPDH, and β-actin were obtained from CST (Beverly, USA). After primary antibody incubation, the membranes were treated with a secondary antibody (1:5000, Beyotime, Beijing, China). Protein bands were detected using enhanced chemiluminescence, with β-actin and GAPDH used as loading controls. The protein expression levels were quantified densitometrically using ImageJ software (NIH, USA).

Statistical analysis

The data obtained were based on SPSS statistical analysis. The continuous data are averagely added and subtracted, and the standard deviation is taken. Statistical analysis, including t tests, one-way ANOVA, and LSD tests, was statistical difference between the two groups ($P < 0.05$).

Results

RNA sequencing (RNA seq) revealed the significantly changed gene in esophageal cancer tissue

Firstly, we applied RNA seq to analyze the significantly changed gene between esophageal cancer tissue and paracancer tissues in humans. We found a high correlation among the 4 esophageal cancer tissue samples (correlation coefficient > 0.4), as well as the 4 paracancer tissue samples (correlation coefficient > 0.55) (Fig. 1A). Figure 1B showed the significantly upregulated or downregulated genes in esophageal cancer tissues. After performing KEGG enrichment analysis, it was observed that the DEGs were mainly enriched in the PI3K-Akt signaling pathway (Fig. 1C). Following the GO analysis, we identified that the differentially expressed genes (DEGs) were primarily enriched in important biological processes, such as cellular processes and biological regulation, key cellular components like cellular anatomical entities, and molecular functions, including binding (Fig. 1D). Based on the results of RNA seq, we focused on the significantly changed gene NEK7 (Log2FC > 1). Next, we verify the level of NEK7 in esophageal cancer tissue and paracancer tissues from 4 patients by qRT-PCR. NEK7 expression was upregulated in esophageal cancer tissues (Fig. 1E). Then, we focused on the role of NEK7 in the development of esophageal cancer in the following study.

The effects of knockdown NEK7 on cell viability, apoptosis, migration, and invasion in human esophageal cancer cell lines

As shown in Fig. 2A, the level of NEK7 was higher in TE-1, Eca109 and EC109 cells than that in HET-1A cells, and its level in EC109 was the highest among the three human esophageal cancer cell lines. Thus, EC109 was selected as the in vitro model of esophageal cancer in following studies. After transfecting with sh-NEK7, the level of NEK7 was reduced in EC109 cells (Fig. 2B). We found cell viability was inhibited in sh-NEK7 group (Fig. 2C). sh-NEK7 significantly promoted EC109 cell apoptosis (Fig. 2D). The results indicated that the migration rate was notably lower in the sh-NEK7 group when compared to the control group (Fig. 2E). As shown in Fig. 2F, we also found cell invasion was reduced after transfecting with sh-NEK7. Knocking down NEK7 in EC109 cells notably

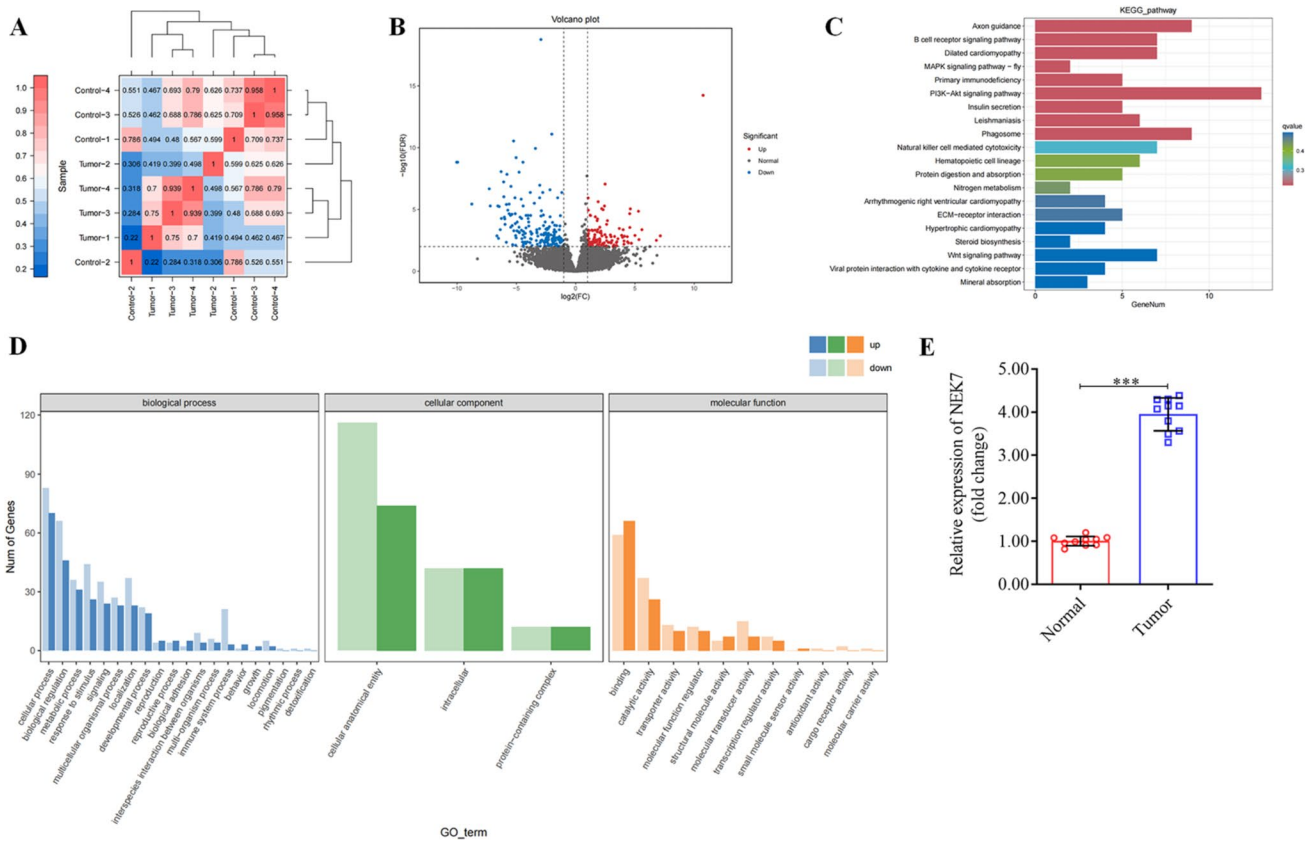


Fig. 1 RNA sequencing (RNA seq) revealed the significantly changed gene in humans. **A** Correlation analysis of samples in esophageal cancer tissue and paracancer tissues. **B** Volcano plot of DEGs. **C** KEGG pathway enrichment of DEGs. **D** GO analysis of DEGs. **E** qRT-PCR was applied to analyze the expression of NEK7 in esophageal cancer tissue. $^{***}P < 0.001$

decreased cell viability, enhanced apoptosis, and inhibited cell migration and invasion, implying that NEK7 is essential for the development of esophageal cancer.

The effect of NEK7 on immune infiltration in esophageal cancer

Then, we asked how NEK7 affect immune infiltration in esophageal cancer. Immune cell infiltration was analyzed by TIMER database. The level of NEK7 showed positive correlation with Tregs ($r = 0.243$, $p = 1.03\text{e-}03$, Fig. 3A), Macrophage M2 ($r = 0.209$, $p = 4.81\text{e-}03$, Fig. 3B), Macrophage M1 ($r = 0.002$, $p = 9.80\text{e-}01$, Fig. 3C), Macrophage ($r = 0.233$, $p = 1.64\text{e-}03$, Fig. 3D), Neutrophil ($r = 0.199$, $p = 7.42\text{e-}03$, Fig. 3E), and Monocyte ($r = 0.153$, $p = 4.04\text{e-}02$, Fig. 3F). NEK7 expression correlates positively with the infiltration of various immune cells, highlighting its potential role in immune modulation in esophageal cancer.

NEK7 binding with NLRP3 and promotes PD-L1 expression

To deeply reveal the mechanism of the role of NEK7 in the development of esophageal cancer, we further to study the interaction between NEK7 and NLRP3. Firstly, interactions between NEK7 and NLRP3 were confirmed by Co-IP (Fig. 4A). Western blot analysis was used to assess the protein expression levels of NLRP3 and PD-L1. While the expression of NLRP3 showed no significant change, a reduction in PD-L1 expression was observed in the sh-NEK7 group (Fig. 4B). In EC109 cells transfected with sh-NLRP3, PD-L1 levels were lowered following the overexpression of NEK7 (Fig. 4C).

The effects of knockdown NEK7 on cell viability, apoptosis, migration and invasion of mEC25 cells

To deeply study the role of NEK7 on cell functions, we further observed the effects of sh-NEK7 on cell viability, apoptosis, migration and invasion. The level of NEK7 and PD-L1 was decreased by sh-NEK7 both in mRNA and protein level

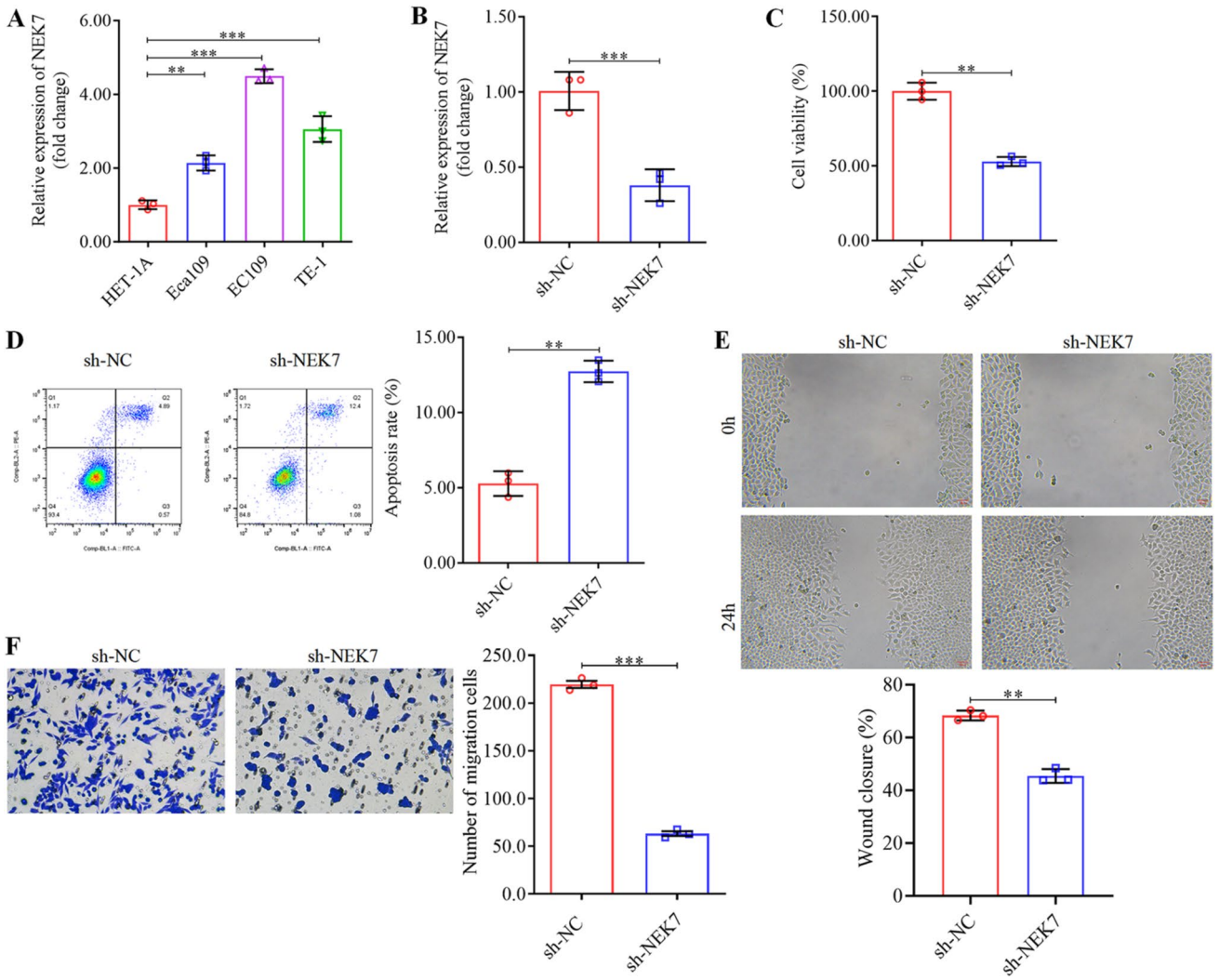


Fig. 2 The effects of knockdown NEK7 on cell viability, apoptosis, migration, and invasion in vitro. **A** qRT-PCR was applied to detect the expression of NEK7 in vitro. **B** The expression of NEK7 in EC109 cells by qRT-PCR. **C** Cell viability by MTT assay. **D** Flow cytometry was used to analyze cell apoptosis. **E** Cell migration by scratch test. **F** Cell invasion by Transwell assay. $^{**}P<0.01$, $^{***}P<0.001$

(Fig. 5A). Cell viability was inhibited in mEC25 cells treated with sh-NEK7 (Fig. 5B). Cell apoptosis was promoted in sh-NEK7 treated cells (Fig. 5C). Cell migration was reduced in sh-NEK7 group (Fig. 5D). As shown in Fig. 5E, we also found cell invasion was inhibited after transfecting with sh-NEK7. In summary, silencing NEK7 in mEC25 cells decreased NEK7 and PD-L1 levels, inhibited cell viability, and reduced migration and invasion.

The effects of knockdown NEK7 on tumor growth and immune cell infiltration in esophageal cancer mice model

Based on the findings in vitro, we established a mouse model to reveal the role of NEK7 in the development of esophageal cancer in vivo. In the esophageal cancer mice model, we observed the size of the tumor was increased, indicating that the in vivo model was successfully established. However, the size of tumor was decreased in sh-NEK7 group (Fig. 6A). Tumor volume was also decreased in sh-NEK7 group (Fig. 6B). We also observed that survival rate was increased mice transfected with sh-NEK7 (Fig. 6C). Knockdown of NEK7 promoted the infiltration of CD4 + T and CD8 + T cells, while inhibiting the infiltration of PD-1 + CD4 + T cells, PD-1 + CD8 + T cells, and Treg cells (Fig. 6D). NEK7 knockdown in the mouse model reduced tumor growth and improved survival, while enhancing T cell infiltration and inhibiting immune suppressive cells.

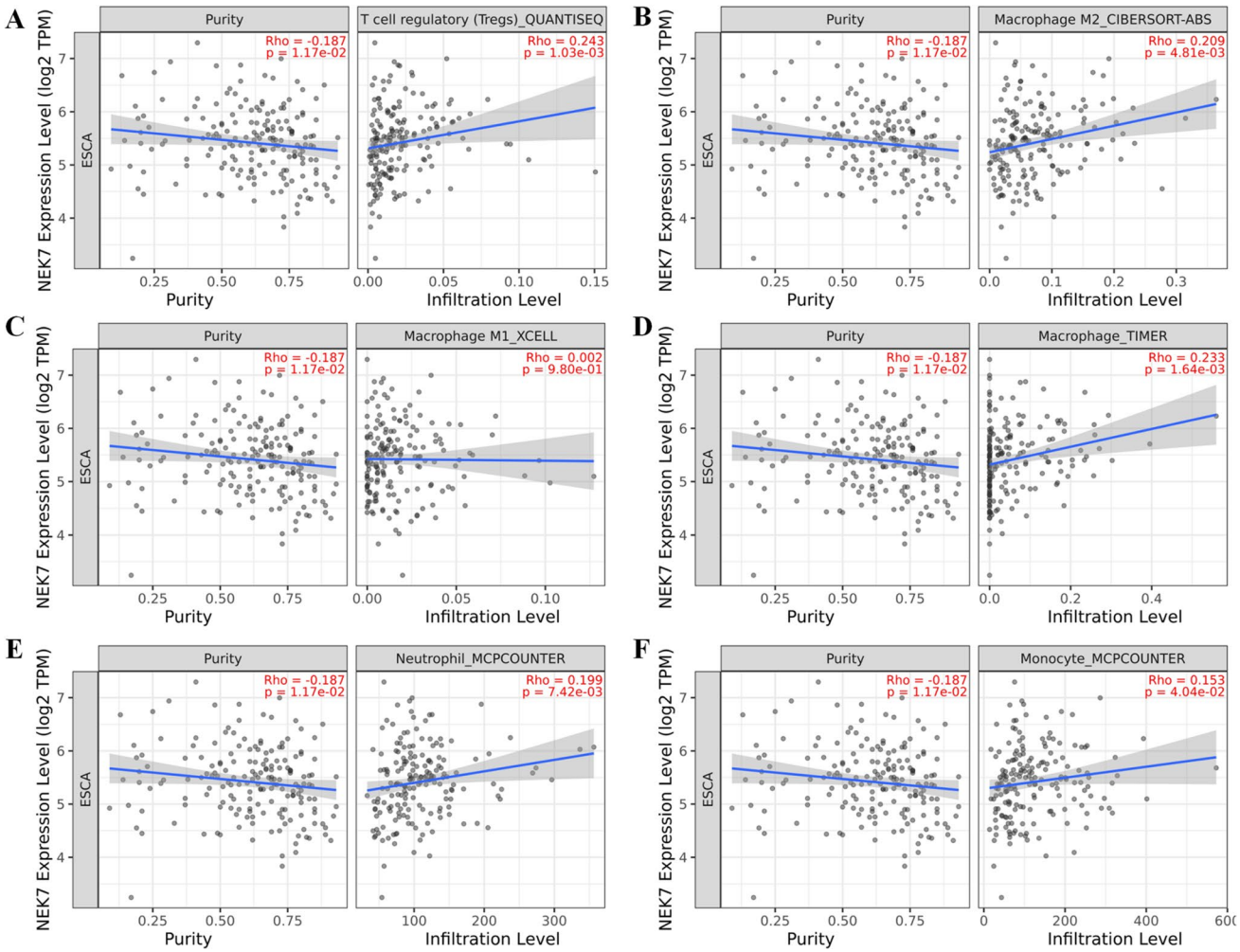


Fig. 3 The effect of NEK7 on immune infiltration in esophageal cancer. Immune Infiltration Analysis in Tregs (**A**), Macrophage M2 (**B**), Macrophage M1(**C**), Macrophage (**D**), Neutrophil (**E**), and Monocyte (**F**) by TIMER database

Effects of licochalcone B on NEK7, NLRP3, PD-L1 expression, cell viability, and tumor cell killing rate

The expression of NEK7, NLRP3, and PD-L1 in EC109 cells with different treatments was measured by Western blot. We found that Licochalcone B had no effects on the level of NEK7 and NLRP3, while it decreased the level of PD-L1. Besides, sh-NEK7 treatment inhibited the NEK7 and PD-L1 expression (Fig. 7A). Licochalcone B blocked the interactions between NEK7 and NLRP3 were confirmed by Co-IP (Fig. 7B). Cell viability was inhibited in Licochalcone B group, as well as the sh-NEK7 group (Fig. 7C). The activity of co-cultured CD8 + T cells was found to be enhanced in both the Licochalcone B group and the sh-NEK7 group (Fig. 7D). In addition, the killing rate of tumor cells was significantly increased in Licochalcone B + anti-PD-1 group (Fig. 7E). Licochalcone B reduced PD-L1 expression, inhibited cell viability, enhanced CD8 + T cell activity, and increased tumor cell killing.

Discussion

The occurrence and death rates of esophageal cancer have been rising annually [17]. Timely detection and treatment are critical for improving the survival rate of patients diagnosed with esophageal cancer. As a result, it is crucial to conduct further research into the root causes of this disease. Gene therapy, as mentioned earlier, is one approach being explored to develop more effective and targeted treatments for esophageal cancer. This therapy directly kills them or make them more susceptible to other treatments [18].

RNA seq has been widely used in gene expression analysis in cancers, including esophageal cancer [19]. However, there is limited research specifically linking the

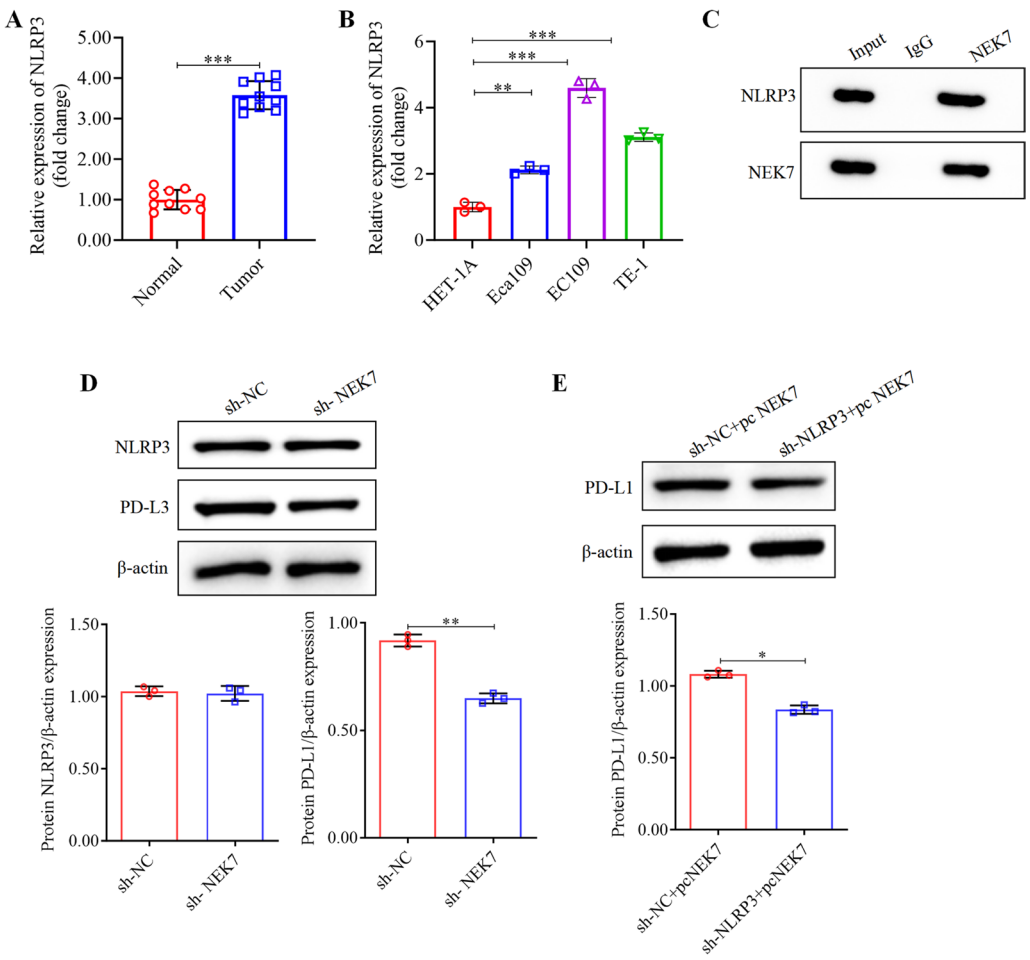


Fig. 4 NEK7 binding activates NLRP3 and promotes PD-L1 expression. **A** The interaction between of NEK7 and NLRP3 by Co-IP. **B** Western blot was applied to analyze the expression of NLRP3 and PD-L1. **C** The expression of PD-L1 by Western blot. $^{*}P<0.05$, $^{**}P<0.01$

NEK7 gene to esophageal cancer. In our study, we used RNA sequencing to analyze gene expression changes in esophageal cancer patients. Our findings revealed that NEK7 was significantly increased in esophageal cancer tissues, which was further confirmed by Real-time PCR analysis. These findings corroborate previous research demonstrating the upregulation of NEK7 in esophageal cancer [12]. HET-1A [20], TE-1 [21], Eca109 [22], and EC109 [23] are common in vitro model in studying esophageal cancer. Here, we also found that the expression of NEK7 was increased in the above esophageal cancer cell lines, especially in EC109. Thus, we selected EC109 cells as the in vitro model in the following experiments. All these results indicated that upregulation of NEK7 was observed in esophageal cancer in vitro and in humans.

Cell proliferation, apoptosis, migration, and invasion are critical cellular processes implicated in the pathogenesis of esophageal cancer [24]. Our study revealed that suppressing NEK7 could effectively hinder cell proliferation, migration, and invasion, while stimulating apoptosis. These findings indicate that targeting NEK7 may hold potential as a therapeutic approach to impede the progression of esophageal cancer in both human and murine models. The tumor immune microenvironment is crucial in the progression of esophageal squamous cell carcinoma [25]. Here, we found that the level of NEK7 showed positive correlation with immune cell infiltration in esophageal cancer. Furthermore, knockdown of NEK7 significantly reduced the size and volume of esophageal cancer tissue and inhibited immune evasion in the tumor microenvironment. This resulted in improved survival rates in the esophageal cancer model mice. These results provide additional evidence supporting the detrimental role of NEK7 in the tumor microenvironment of esophageal cancer and its promotion of malignant development.

It has been demonstrated the critical involvement of NEK7 in the activation of the NLRP3 inflammasome through its interaction with neighboring NLRP3 subunits [26]. Thus, lots of studies showed the NEK7-NLRP3 interaction in studying different diseases [27], including

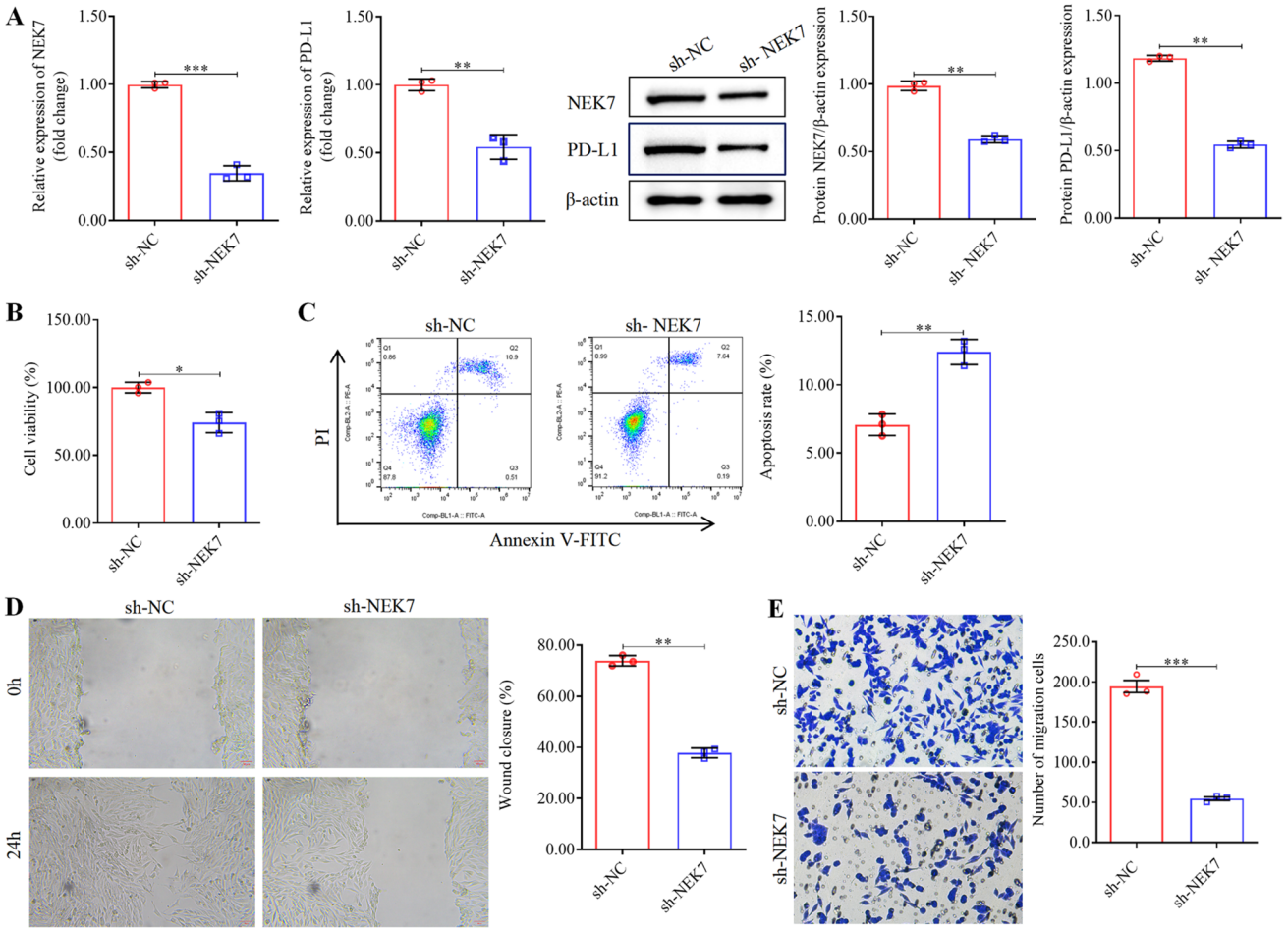


Fig. 5 The effects of knockdown NEK7 on cell viability, apoptosis, migration and invasion in mEC25 cells. **A** The expression of NEK7 and PD-L1 by qRT-PCR and Western blot. **B** MTT assay used to detect cell viability. **C** Cell apoptosis by flow cytometry. **D** Cell migration by scratch test. **E** Transwell assay was used to measure cell migration invasion. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

cancer [28]. We observed an interaction between NEK7 and NLRP3 in our in vitro experiments, which is consistent with previous reports. Furthermore, our study revealed that inhibiting the PD-L1/PD-1 signaling pathway could augment the immune response against the tumor, which was consistent with the findings in previous study [29]. We further explore the regulated effects of NEK7 on NLRP3 and PD-L1. Sh-NEK7 had no effects on the level of NLRP3, but it decreased the level of PD-L1. In sh-NLRP3 treated cells, we also found that the level of PD-L1 was decrease after transfecting with pc-NEK7. All these results indicated that NEK7 and NLRP3 had interaction, and they all played the positive role in regulating PD-L1 both in EC109 and mEC25 cells.

Licochalcone B, a natural compound, demonstrates significant anti-tumor activity by modulating immune responses and cell viability in esophageal cancer (EC) cells [30]. In the current study, Licochalcone B was found to decrease the expression of PD-L1 without affecting NEK7 or NLRP3 levels. PD-L1, an immune checkpoint protein, plays a crucial role in immune evasion by inhibiting CD8 + T cell activity. By reducing PD-L1 expression, Licochalcone B likely enhances T cell-mediated tumor cell killing, as evidenced by the increased activity of co-cultured CD8 + T cells. Further, the sh-NEK7 treatment also reduced NEK7 and PD-L1 expression, suggesting that NEK7 contributes to immune suppression through regulation of PD-L1. NEK7, a key inflammasome component, has been implicated in modulating immune responses and maintaining tumor cell survival [31]. Its interaction with NLRP3 was disrupted by Licochalcone B, supporting the idea that Licochalcone B can block inflammasome activation, which is often linked to immune evasion and tumor progression. Both Licochalcone B and sh-NEK7 inhibited cell viability, further indicating the anti-tumor potential of these treatments. Importantly, the combination of Licochalcone B and anti-PD-1 therapy significantly enhanced tumor cell killing, highlighting

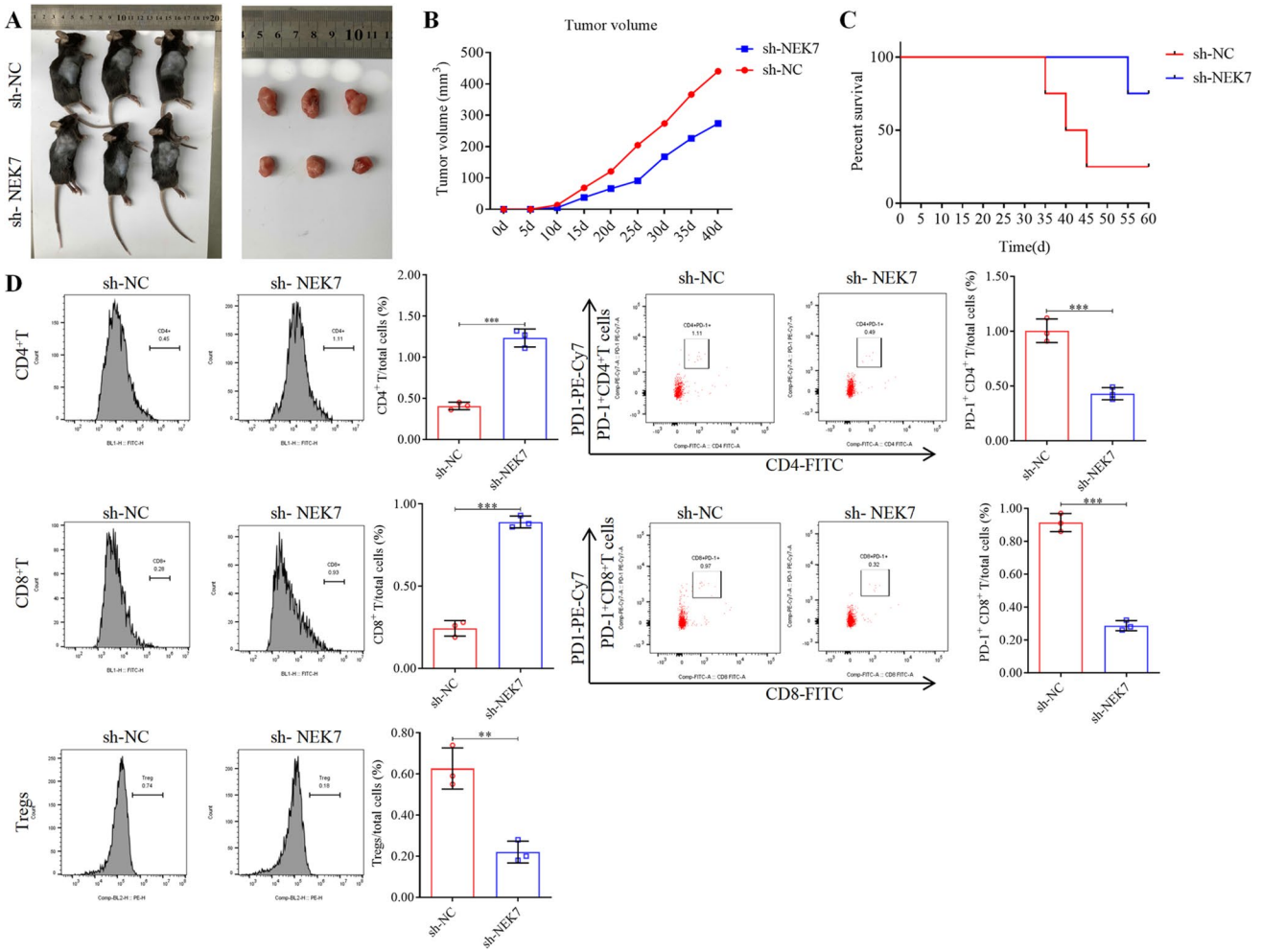


Fig. 6 The effects of knockdown NEK7 on tumor growth in esophageal cancer mice model. **A** Representative image of esophageal cancer mice and tumor size. **B** Tumor volume. **C** Survival analysis. **D** Immune cells infiltration analysis by flow cytometry. $^{**}P<0.01$, $^{***}P<0.001$

the therapeutic potential of Licochalcone B in boosting immune responses and improving cancer treatment efficacy.

For the strengths of this study, this study reveals NEK7 as a critical regulator in esophageal cancer progression and immune evasion, highlighting its potential as a therapeutic target through modulation of the NEK7/NLRP3 pathway. For the limitations of this study, the study mainly focuses on NEK7 and immune modulation, but the exact molecular mechanisms remain unclear. The clinical relevance and safety of Licochalcone B are not evaluated, requiring further clinical trials for validation.

In conclusion, our study highlights the critical role of NEK7 in the progression of esophageal cancer, influencing cell viability, apoptosis, migration, and immune infiltration. Targeting NEK7, particularly through Licochalcone B, offers a promising therapeutic strategy by modulating immune responses and enhancing tumor cell killing, potentially improving cancer treatment outcomes.

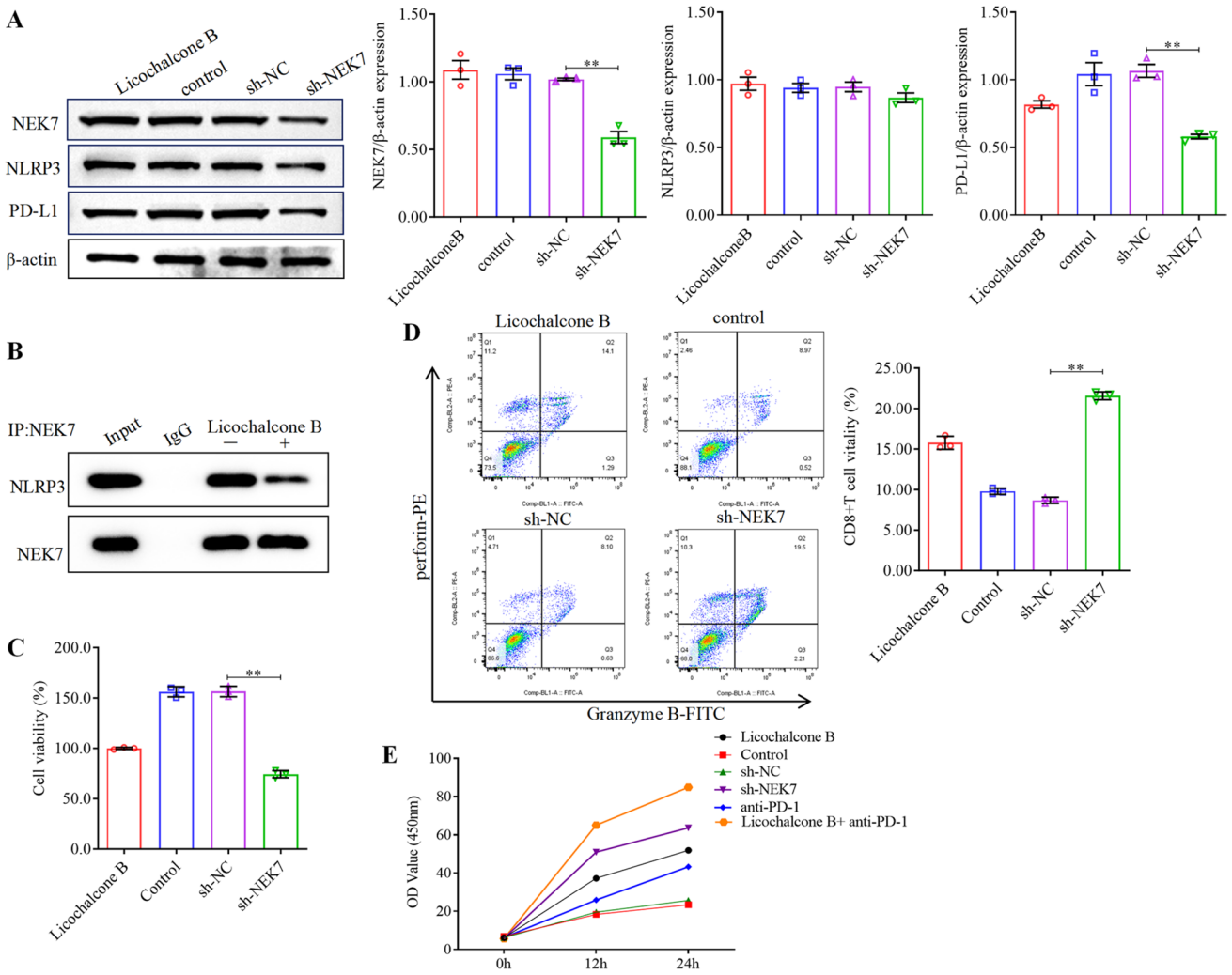


Fig. 7 The effects of Licochalcone B on the expression of NEK7, NLRP3, and PD-L1, cell viability and killing rate of tumor cells. **A** The expression of NEK7, NLRP3, and PD-L1 by Western blot. **B** Co-IP. **C** MTT assay. **D** CD8 + T cell activity by flow cytometry. **E** Long-term (12 and 24 h) killing assay. $^{**} < 0.01$

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent participate The experimental protocols were approved by the Medical Ethics Committee of Beijing Luhe Hospital, Capital Medical University (Approval number: 2023-LHKY-019–02). The patients/participants provided their written informed consent to participate in this study.

Human and animal rights All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care and Use guidelines of Tsinghua University, China, and approved by the Institutional Animal Care and Use Committee of Tsinghua University in China (Approval number: THU-LARC-2024–019).

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