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ORIGINAL RESEARCH

ESAT-6 Modulates Macrophage Apoptosis in Mycobacterium Tuberculosis via lncNEAT1/miR-125b-5p/TNF-α Pathway

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Introduction: Tuberculosis (TB), resulting from the bacterial pathogen Mycobacterium tuberculosis (Mtb), continues to be a leading cause of death and illness globally. Mtb employs secretory proteins to avoid host immune responses during the infection process and is able to survive, spread and replicate within the hostile micro-environment. Early secreted antigenic target 6 kDa (ESAT-6), the major virulence factor of Mtb, plays an important role in Mtb-induced macrophage apoptosis, which could benefit the dissemination of Mtb. However, the underlying mechanism of ESAT-6 in macrophage apoptosis still unclear.

Methods: In this research, the human monocytic leukemia cell lines (THP-1) were treated with Phorbol 12-myristate 13-acetate (PMA) to differentiation into M_0 macrophages, and the role of recombinant ESAT-6 in macrophage apoptosis was investigated using Annexin V-FITC/propidium iodide (PI) assay with Flow CytoMetry analysis. The small interfering RNA (siRNA) of Long non-coding RNA nuclear enriched abundant transcript 1 (lncNEAT1) was used to silencing the RNA expression of lncNEAT1. The expression level of lncNEAT1, microRNA-125b-5p (miR-125b-5p) and tumor necrosis factor-alpha (TNF-α) mRNA were investigated using RT-qPCR technique. Additionally, Western blot was used to detect the protein expression of TNF-α. Furthermore, the downstream regulating mechanisms of lncNEAT1 were investigated using bioinformatics analyses and luciferase reporter assays.

Results: The results showed that ESAT-6 induces macrophage apoptosis in a dose-dependent manner via upregulation of the lncNEAT1 and lncNEAT1 can target miR-125b-5p, while miR-125b-5p can also target the 3′UTR (Untranslated Regions) of TNF-α mRNA. Moreover, inhibition of lncNEAT1 alleviated ESAT-6 induced macrophage apoptosis by targeting miR-125b-5p/TNF-α axis.

Discussion: The results of this study indicated that ESAT-6 induces macrophage apoptosis by regulating lncNEAT1/miR-125b-5p/TNF-α pathway, which may provide a possible therapeutic target for the treatment of TB.

Keywords: apoptosis, ESAT-6, macrophage, Mycobacterium tuberculosis, tuberculosis

Introduction

Tuberculosis (TB), an infectious disease caused by Mycobacterium tuberculosis (Mtb), continues to be a major global health issue due to its high rates of morbidity and mortality. The WHO Global Tuberculosis Report 2024 indicates that, even with progress in tuberculosis prevention and control, Mtb has infected more than 10.8 million people and caused 1.25 million deaths in 2023.[1] The pathogenic success of Mtb is primarily attributed to its ability to manipulate the host's innate immune defenses, enabling it to persist, disseminate, and replicate inside the harsh conditions of macrophages and other cellular niches.

TB primarily involves the lungs, thus causing pulmonary TB (PTB), however it can also affect new sites like pleural cavities, meninges, lymph nodes, urogenital organs, bone and joints, leading to extrapulmonary TB (EPTB).[2] In PTB, macrophages serve as the initial immune defense barrier not only as a site for Mtb to grow within cells but also as key cells that present mycobacterial

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antigens, initiating inflammation, breaking down bacilli, and stimulating adaptive immune responses.[3] Macrophages identify Mtb via pattern recognition receptors (PRRs), triggering the release of pro-inflammatory cytokines like TNF, interleukin-12 (IL-12), and IL-1β, which attract adaptive immune cells to assist in the mycobactericidal response.[4] In addition, Mtb infection causes macrophages to undergo transcriptome reprogramming, altering gene expression related to immunity and resisting the innate immune system, which affects the survival and dissemination of Mtb.[5] Mtb possesses an early secretory antigenic target secretion (ESX) system, and the ESX-1 system is required to permeabilize the phagosomal membrane, thereby enabling tuberculosis necrotizing toxin (TNT) trafficking to the cytosol and subsequent escape of Mtb from the phagosome and, eventually, from the dying macrophage.[6] ESAT-6 (Rv3875), a 6 kDa early secreted antigenic target, is delivered through the ESX-1 system and regulates Mtb's colonization, survival, pathogenesis, and granuloma formation.[7] ESAT-6 is encoded in a region of difference (RD) 1, which was absent from Mycobacterium bovis (M.bovis), Bacillus Calmette-Guerin (BCG), Non-tuberculous Mycobacteria (NTM) and most environmental Mycobacteria, and ESAT-6 used for stimulating the lymphocytes in Interferon-gamma Release Assay (IGRA) test as a specific antigen detection for distinguishing Mtb infection from previous BCG vaccination or NTM exposure.[8] Additionally, ESAT-6 is the key player responsible for the pathogenicity of Mtb and is present only in the virulent strains of Mtb.[7,9] Serves as a major virulence factor and a key antigen of Mtb, ESAT-6 is described as a pro-apoptotic factor that aids in the dissemination of Mtb.[10,11] The first demonstration of ESAT-6 causing apoptosis in macrophages was reported by Derrick et al in 2007.[12] It has been observed that ESAT-6 not only increases the expression of caspases-3, caspases-5, caspases-7, and caspases-8 in THP-1 cells[12] but also causes endoplasmic reticulum stress and enhances the activation of caspases-12 and caspases-3 in A549 cells,[13] reduces BCL-2, and enhances caspase-3 expression in macrophages.[14] It was suggested by these findings that ESAT-6 is a key factor in the process of Mtb-induced apoptosis. In addition, studies have showed[14–16] that compared to the attenuated Mtb strain, the virulent Mtb strain is a stronger inducer of macrophage apoptosis, implying that apoptosis might support Mtb dissemination, with ESAT-6 inducing macrophage apoptosis and possibly enhancing extracellular transmission of Mtb. However, the mechanism of ESAT-6 induced macrophage apoptosis remain unclear.

Long non-coding RNAs (lncRNAs) are a string of RNA molecules that longer than 200 nucleotides (nt) and not translated into protein products, which involved in a variety of key biological processes and human diseases, including cancer and inflammatory diseases.[17,18] Growing evidence suggests that lncRNAs play a role in regulating the host's immune system and are linked to TB outcomes.[17,19,20] Li et al discovered that the reduction of lncPCED1BAS1 was linked to the progression of active TB and confirmed that lncPCED1BAS1 regulated the macrophage apoptosis via miR-155 axis in active TB.[19] Recently, Wang et al discovered that the expression level of Long non-coding RNA nuclear enriched abundant transcript 1 (lncNEAT1) was significantly increased in the peripheral blood and granulomatous tissue of individuals with spinal tuberculosis (STB) as well as in THP-1 cell lines infected with Mtb.[17] Huang et al showed that lncNEAT1 is highly expressed in the peripheral blood of TB patients and is related to the prognosis of TB.[20] Notably, studies have reported that lncNEAT1 plays a role in cell proliferation and migration across different human cancers, including endometrial,[21] breast,[22] and cervical cancers.[23] In previous studies,[24,25] lncNEAT1 was reported to act as a regulator of micro-RNA (miRNA) and to play an important role in cellular processes, including apoptosis. Dong et al reported that lncNEAT1 promotes apoptosis in N-methyl-4-phenylpyridinium (MPP+)-treated SH-SY5Y cells and inhibiting lncNEAT1 effectively promotes cell growth, reduces apoptosis, and improves the viability of SH-SY5Y cells by targeting miR-374c-5p.[24] According to Liu et al, lncNEAT1 enhances apoptosis and inflammation in H9c2 cells stimulated with Lipopolysaccharide (LPS) by acting as a sponge for miR-590-3p.[25] According to reports, lncNEAT1 can target the downregulation of miR-125b-5p expression, and by which effects on the human retinal microvascular endothelial cells (hRMECs) proliferation and migration,[26] as well as promotes apoptosis of the T lymphocyte.[27] In addition, it has been shown that miRNA binding to the 3′UTR of the target mRNA results in mRNA cleavage or inhibition of translation.[28,29] Many studies have shown that miR-125b-5p targets the 3′UTR of TNF-α transcripts and play a regulatory role in production of pro-inflammatory cytokines.[30,31] It is well known that, TNF-α is a pro-inflammatory cytokine associated with macrophage apoptosis and inflammatory response during the process of Mtb infection.[32–34] From above study, it would be concluded that lncNEAT1 could play a role in regulating the innate immunity and is associated with the progression of TB via lncRNA-miRNA-mRNA regulatory networks.

Overall, existing studies have shown the role of lncNEAT1 as a gene regulator in the process of cell apoptosis and its association with the occurrence of TB. However, regulatory networks and underlying functions of lncNEAT1 in ESAT-6 induced macrophage apoptosis have rarely been investigated. In this study, the role of ESAT-6 on macrophage apoptosis

by upregulating of lncNEAT1 and regulatory function of lncNEAT1 in ESAT-6 induced macrophage apoptosis by targeting the miR-125b-5p/TNF-α axis were verified. The present study aimed to elucidate the potential mechanism of ESAT-6 induced macrophage apoptosis and the regulatory networks of lncNEAT1 in ESAT-6 induced macrophage apoptosis for identify possible targets for TB treatment.

However, because of the source constraints of primary macrophages, which causes the limitations on macrophage-related studies. Furthermore, THP-1 cells serve as an excellent model system in macrophage studies because they can differentiate into M_0 type macrophages using phorbol 12-myristate 13-acetate (PMA) stimulation.[35] As reported by Mohd et al, macrophages derived from THP-1 cells are frequently utilized as substitutes for human primary macrophages, and this study mentioned that from 2010 to 2020, PMA served as a stimulant for differentiating THP-1 cells into M0 macrophages in 85 articles.[36] Therefore, PMA-stimulated THP-1 cells were used in this study to examine the role of ESAT-6 in macrophage apoptosis.

Materials and Methods

Cell Culture and Treatment

The THP-1 cell line was obtained from Pricella (CL-0233, Pricella Inc., China). Cell culture media (Advanced RPMI 1640), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and cell culture antibiotics (penicillin/streptomycin) were obtained from Gibco (Thermo Fisher). To establish the THP-1 cell line as a human macrophage model, 100 ng/mL of PMA (TQ0198, TargetMol Topscience, China) were added to the 1×10^6/plate of THP-1 cells, and cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Next, macrophages underwent treatment with ESAT-6 at concentrations of 4/8/12 μg/mL for 12 h. Full-length recombinant ESAT-6 protein was obtained from Abcam (ab124574, Abcam) and dissolved in PBS at a concentration of 10 μg/mL. Macrophages were treated with 100 nM si-NEAT1 (Thermo Fisher) or Silencer™ Select Negative Control siRNA for 24 h (4390843, Thermo Fisher) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher).

Apoptosis Assay

After washing with PBS, macrophages were stained by Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit for 15 min (Thermo Fisher) and the apoptotic cells detected by Flow CytoMetry assay according to the manufacturer's protocol. The FlowJo software (Tree Star, USA) was used to analyze the results, and the stained cells were counted to calculate the percentage of apoptotic cells. The experiments were repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RIPA reagent (Thermo Fisher) was used to extract the total RNAs from cultured cells, and Revert Aid RT kit (Thermo Fisher) was used to reverse transcription for total RNAs to complementary DNA (cDNA). In the next step, SYBR Premix Ex Taq™ (Takara Bio) was used to performing qPCR experiment by CFX96 Real-Time PCR Detection System (Bio-Rad). The expression levels of lncNEAT1, miR-125b, and TNF-α were calculated using the $2^{-\Delta\Delta Ct}$ method, with the small non-coding RNA U6 and GAPDH as the respective internal controls. The experiments were repeated three times using the following primers:

GAPDH:
forward:5-CAGGAGGCATTGCTGATGAT-3,
reverse:5-GAAGGCTGGGGCTCATTT-3.
lncNEAT1:
forward:5-GCCACAACGCAGATTGATGC-3,
reverse: 5- AGGCAAACAGG-TGGGTAGGT-3.
miR-125b:
forward:5-CTGATAAATCCCTGAGACCCTA-3,
reverse:5- ATGGTTTTGACGACTGTGTG-3.
U6:
forward:5-CTCGCTTCGGCAGCACA-3,
reverse:5-TGGTGTCGTGGAGTCG-3.

TNF-α:
forward:5-CACACACACCCTCCTGATTG-3, reverse:5-CTCATTCAACCCTCGAAAA-3.

Luciferase Assay

A luciferase reporter assay was performed by co-transfection of pLUC-REPORT vector (Ambion, USA) containing wild-type or mutant 3′-UTR of TNF-α mRNA or lncNEAT1 containing the mirVana™ miRNA Mimic Negative Control (4464059, Thermo Fisher) and miR-125b-5p mimic (Thermo Fisher) binding site into HEK-293T cells (CL-0005, Pricella Inc., China) in 96-well plates using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions. After 48 hours of transfection, the Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, USA) using SpectraMax M5 (Molecular Devices, USA).The potential target miRNAs of lncNEAT1 was predicted using DIANA-LncBase v3.0 (https://diana.e-ce.uth.gr/lncbasev3) and the potential target genes of miRNAs were predicted using TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org).

Western Blot Analysis

The protein samples were extracted from THP-1 cells with RIPA lysis buffer, and the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). The separation of 30 μg of total protein per lane was achieved with 10% SDS-PAGE and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (EMd Millipore, USA) followed by protocols. At room temperature, the PVDF membranes underwent a 2-hour blocking process with 5% bovine serum albumin (BSA, Solarbio) at room temperature. In the next step, PVDF membranes were stored overnight at 4 °C for incubated with a primary antibody against TNF-α (ab183218, 26 kDa; 1:500; Abcam) and then washed three times with TBST, each wash lasting 10 minutes. Then, the PVDF membranes were incubated for 1 h with Goat anti-rabbit IgG secondary antibody (ab97080;contains HRP; 1:2000; Abcam) at room temperature. Finally, the Pierce^{TM} ECL (chemiluminescence) Western buffer (Thermo Fisher) was used to visualized the protein bands. GAPDH (ab8245; 1:1000; Abcam) was used as an internal control. The experiments were repeated three times.

Statistical Analysis

Statistical analyses were performed using SPSS 26.0, and GraphPad Prism v8.0 software was selected to construct graphs. The results are presented as means±SEM, and all experiments were implemented at least 3 times. Student's *t*-test or One-way ANOVA method was used to estimate the significance of the statistical results. For p-values, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, in graphs "*", "**" and "***"denote significant differences.

Results

To examine the role of ESAT-6 in macrophage apoptosis, THP-1 cells were treated with PMA for differentiated into macrophages according to previous studies. The macrophages were treated with different concentrations of ESAT-6 to examine its role of ESAT-6 in the modulation of macrophage apoptosis. In addition, as a parallel experiment, ESAT-6 treated macrophages were co-treated with the small interfering RNA of lncNEAT1 (siNEAT1) for inhibition RNA expression of lncNEAT1 to examine the role of lncNEAT1 in the modulation of ESAT-6 induced macrophage apoptosis. The levels of apoptosis and RNA expression of lncNEAT1 in macrophages were detected by Flow Cytometry and RT-qPCR at 12 h post-infection. We found that ESAT-6 treatment significantly induced apoptosis in macrophages compared to untreated control macrophages, the percentage of apoptosis in the control group was 5.65±0.16, in the 4μg/mL of ESAT-6 treated group was 14.68±0.38, in the 8μg/mL of ESAT-6 treated group was 25.45±0.33, in the 12μg/mL of ESAT-6 treated group was 34.48±0.54. In stark contrast, treatment with siNEAT1 significantly inhibited ESAT-6 induced macrophage apoptosis compared to siNEAT1 untreated groups (Figure 1A and B), the percentage of apoptosis in the siNEAT1 + control group was 5.87±0.37, in the siNEAT1 + 4μg/mL of ESAT-6 treated group was 13.78±0.16, in the siNEAT1 + 8μg/mL of ESAT-6 treated group was 15.03±0.15, in the siNEAT1 + 12μg/mL of ESAT-6 treated group was 20.25±0.47. In addition, the RNA expression level of lncNEAT1 was significantly up-regulated in ESAT-6 treated macrophages compared to untreated control macrophages, and treatment with siNEAT1 significantly inhibited the RNA

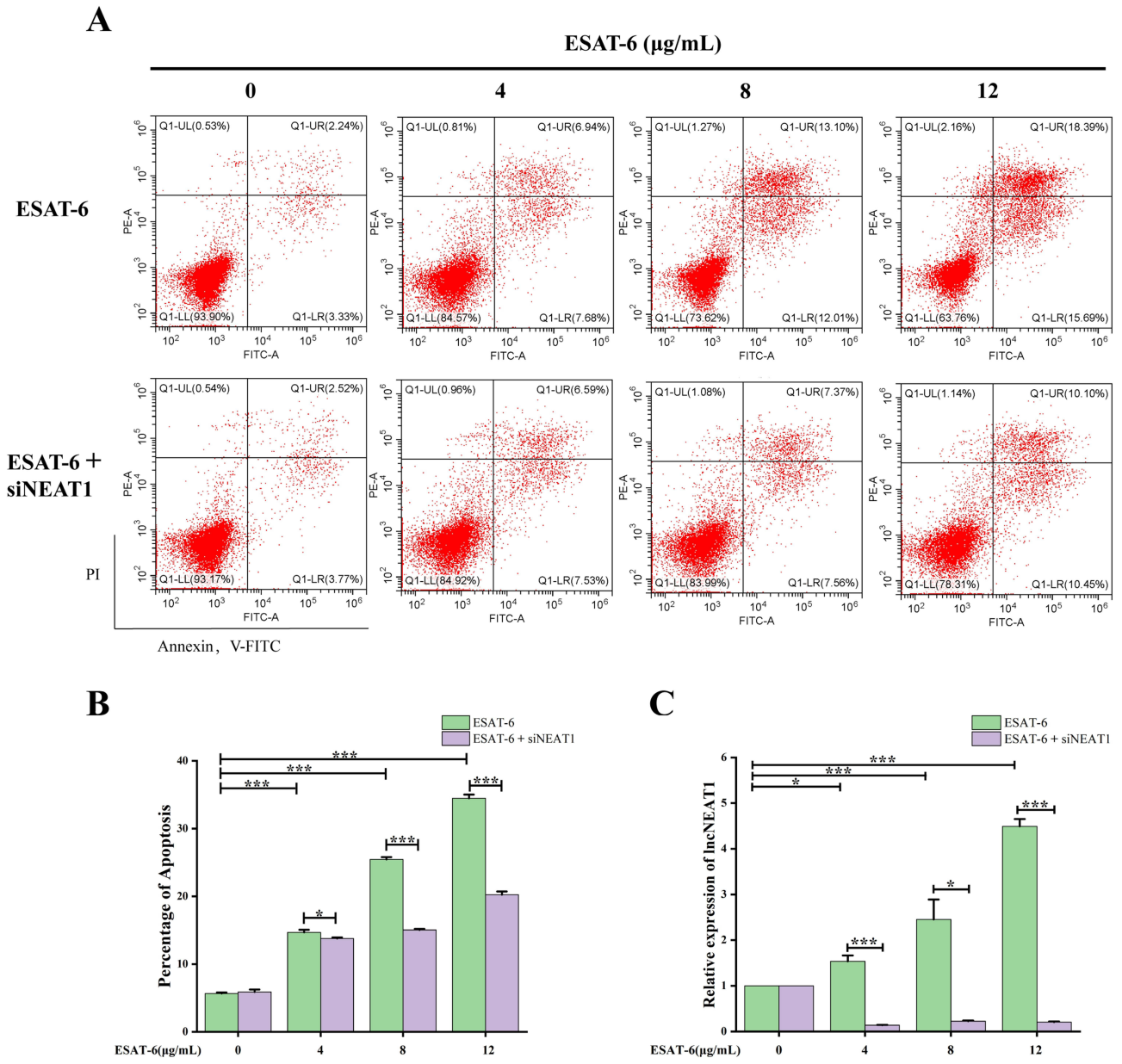

Figure 1 ESAT-6 induces macrophage apoptosis via up-regulation of lncNEAT1. (**A**) Flow cytometric analysis of apoptosis of macrophages. The macrophages were treated with 4 μg/mL, 8 μg/mL, 12 μg/mL concentration of ESAT-6 for 12 h, and other plates of ESAT-6 treated macrophages were co-treated with siNEAT1 in the same time. The flow cytometry plot displayed is typical of three experiments. (**B**) The percentages of apoptotic cells are expressed as means±SEM data, n=3. "*" and "***" denote significant differences ($p < 0.05$ and $p < 0.001$, respectively). (**C**) The qPCR experiments was used to measure the expression of the lncNEAT1 gene. The results are presented as means±SEM, n=3. lncNEAT1 gene expression was normalized to the internal control, GAPDH. "*" and "***" denote significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). One-way ANOVA test was used for comparation between different concentration of ESAT-6 treatment groups and control group, the Student's *t*-test was used for comparation between siNEAT1 treated and untreated groups.

expression of lncNEAT1, which is equivalent to verifying the role of siNEAT1 interference (Figure 1C). These results indicate that ESAT-6 elicits the production of lncNEAT1 in macrophages and ESAT-6 induces apoptosis by regulating lncNEAT1.

To reveal how lncNEAT1 modulates ESAT-6-induced macrophage apoptosis, the potential target miRNAs of lncNEAT1 and potential target genes of the miRNA were predicted using bioinformatics analyses. The predicted informatics showed that lncNEAT1 had binding sites for miR-125b-5p, and the miR-125b-5p sequence exhibited a binding site for the 3′UTR (Untranslated Regions) of TNF-α mRNA (Figure 2A). To further verify the functional interaction between lncNEAT1/miR-125b-5p and miR-125b-5p/TNF-α, the luciferase reporter assays were employed to

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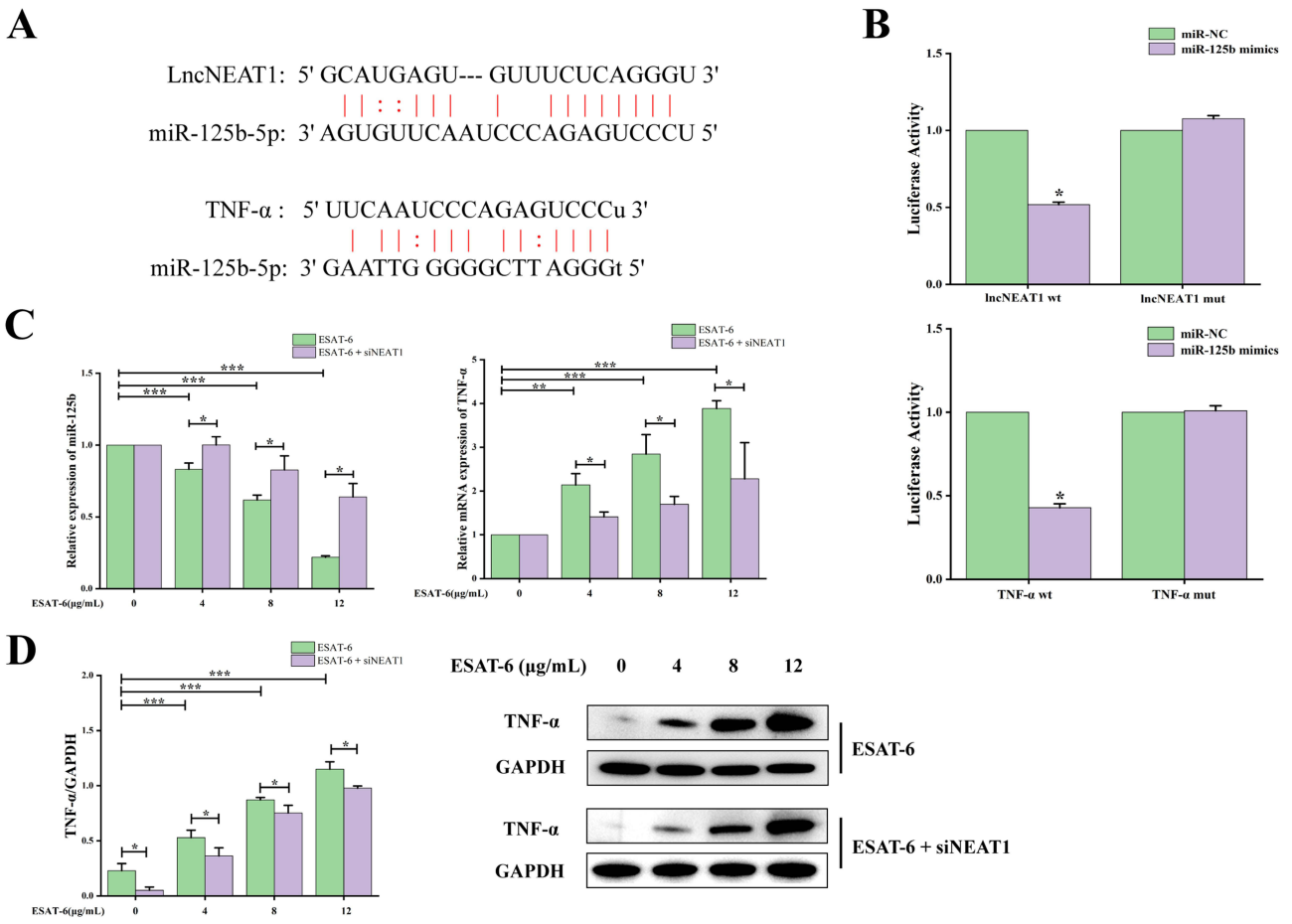

Figure 2 ESAT-6 regulates lncNEAT1/miR-125b-5p/TNF-α pathway. (**A**) According to the base pairing principle, the binding of lncNEAT1 to the predicted sequence of miR-125b-5p was predicted using DIANA-LncBase v3.0 version, there were fourteen complementary sites. The binding of miR-125b-5p to the predicted sequence in the 3′UTR of TNF-α mRNA was predicted using miRanda and TargetScan, there were twelve complementary sites. (**B**) MiR-125b-5p mimics or negative control mimics (miR-NC) and wild-type lncNEAT1 (lncNEAT1 wt) or mutant lncNEAT1 (lncNEAT1 mut) were co-transfected into HEK293T cells. MiR-125b-5p mimics or miR-NC and wild-type 3′UTR (TNF-α wt) or mutant 3′UTR (TNF-α mut) of TNF-α were co-transfected into HEK-293T cells. The luciferase activity was observed 48 hours post-transfection. The results are presented as means±SEM, n = 3. "*" denote significant differences ($p < 0.05$ respectively). Student's *t*-test was used for comparation. (**C** and **D**) The gene expression of miR-125b-5p and TNF-α mRNA were assayed by qPCR. The expression of TNF-α protein was assessed through Western blotting with a TNF-α antibody. The qPCR and blotting data are means±SEM, n=3. The miR-125b-5p gene expression was normalized to the small nuclear RNA control, U6. The mRNA/protein expression of TNF-α gene was normalized to the internal control, GAPDH. "*", "**" and "***" denote significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). One-way ANOVA test was used for comparation between different concentration of ESAT-6 treatment groups and control group, the Student's *t*-test was used for comparation between siNEAT1 treated and untreated groups.

confirm the binding targets. As shown, the luciferase activity of the reporter gene significantly decreased in the miR-125b-5p mimics transfected wild-type genotype (WT) cells, and transfecting with miR-125b-5p mimics did not significantly impact the luciferase activity of the reporter gene in the mutant genotype (MT) cells. The results of bioinformatics analyses and luciferase reporter assays were indicating miR-125b-5p targeted the lncNEAT1 and 3′ UTR of the TNF-α gene (Figure 2B), and verified that lncNEAT1 can target miR-125b-5p, while miR-125b-5p can also target the TNF-α gene.

Based on this, it was found in the previous cell culture that ESAT6 treatment significantly inhibited the expression levels of miR-125b-5p and significantly increased the mRNA/protein expression levels of TNF-α (Figure 2C and D). In addition, lncNEAT1 inhibition markedly reversed the expression of miR-125b-5p and the mRNA and protein expression of TNF-α (Figure 2C and D). Therefore, it was preliminarily determined that ESAT-6 targeted the downstream miR-125b-5p/TNF-α axis by promoting lncNEAT1 expression during ESAT-6 induced macrophage apoptosis.

Discussion

Even with recent progress in experimental medicine, treatment options and preventive strategies for TB are still restricted. There for, more research is needed to understand the mechanism of the extracellular transmission of Mtb. In the present study, our data showed that treatment with ESAT-6 induced apoptosis in macrophages and upregulated the levels of lncNEAT1 in a concentration-dependent manner. Additionally, lncNEAT1 upregulation targeted the miR-125b-5p/TNF-α axis. These results indicate that ESAT-6 induces apoptosis in macrophages by regulating the lncNEAT1/miR-125b-5p/TNF-α pathway, which could be a potential molecular mechanism for the dissemination and extracellular transmission of Mtb.

Apoptosis is the most prominent mode of programmed cell death, and is necessary for maintaining tissue homeostasis. Apoptotic pathways are crucial in Mtb-infected macrophages, and macrophage death is an essential response to TB development.[37] Studies have shown that during the initial phase of infection, macrophage apoptosis is usually regarded as a host defense against Mtb invasion.[38,39] However, during the advanced phases of infection, increased bacterial proliferation leads to excessive apoptosis of macrophages, which increases the secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IFNs, causing macrophage cell death and dissemination of infection.[40,41] In addition, relevant studies have shown that in the advanced phases of lung granulomas, heightened bacterial growth hinders the regression of TB, and an abundance of apoptotic macrophages may promote the spread of Mtb rather than phagocytosis and antigen processing.[42,43] There for, apoptosis of macrophages in granulomas favors the pathogen process, which resulting in bacterial release into the extracellular environment and an open pathway for transmission.[44] In this study, our data showed that ESAT-6 treatment induced macrophage apoptosis in a concentration-dependent manner, indicating that ESAT-6 may play a critical role in macrophage cell death and dissemination of infection.

Mtb is a pathogenic microorganism can regulating the gene expression of the host, which induce infection and inflammatory in the TB lesion, non-coding RNA acts as a crucial regulatory process.[45,46] The up-regulation of lncRNA or reducing of miRNA levels leads to an increase in several downstream targets, causing alterations in immune response, which serves as a crucial regulatory point for non-coding RNA in managing the immune response to bacterial infections. Several studies have shown that Mtb infection leads to changes on the expression of the non-coding RNAs in a ESAT6 dependent way.[10,16] Previous studies have shown that lncNEAT1 is related to Mtb infection[17,20] and plays a significant role in the process of cell apoptosis.[24,25] In our study, lncNEAT1 was verified as an apoptosis-related lncRNA and evaluated its regulatory role in ESAT-6 induced macrophage apoptosis.Then, the downstream targets were predicted using bioinformatics methods, and the relationship between targeted binding was validated using a dual-luciferase reporter assay. The results showed that ESAT-6 elicits the production of lncNEAT1 in THP-1 cells and upregulates lncNEAT1 targeting the miR-125b-5p/TNF-α axis by downregulating the expression level of miR-125b-5p and upregulating the expression level of TNF-α. Notably, previous studies have shown that lncNEAT1 can target the downregulation of miR-125b-5p expression[26,27] and miR-125b-5p targets the 3′UTR of TNF-α transcripts.[28,29] As a pro-inflammatory cytokine, TNF-α plays a critical role in triggering apoptosis in macrophages, which is vital for restricting the intracellular growth of Mtb and aiding its removal by the immune system.[47] Studies demonstrate that TNF-α is involved in the initial immune response during the infection process by its important role in cell apoptosis. Zhou et al reported that TNF-α played a central role in the apoptosis in the context of Angiostrongylus cantonensis infection by regulating RIP1/FADD/Caspase-8 axis.[48] Kumar et al reported that TNF-α promotes cell apoptosis in Aeromonas hydrophila-infected head-kidney macrophages (HKM) by regulating caspase-1/IL-1β inflammatory axis.[49] Upon Mtb infection, key cytokines like TNF-α and IL-1 are involved in the initial immune response, in which TNF-α is a significant pro-apoptotic cytokine that induces macrophage apoptosis and recruits immune cells to the site of infection.[34,50] In addition, previous study have reported that Mtb EsxL ESAT-6 induces TNF-α secretion by interacting with the TLR2 receptor in macrophages, and the production of TNF-α is necessary for the activation of pro-inflammatory responses in macrophages during Mtb infection and in forming granulomas.[51] Similarly, our current data shows that ESAT-6 induced TNF-α secretion as well as macrophage apoptosis in a concentration-dependent manner. Therefore, the lncNEAT1/miR-125b-5p/TNF-α pathway, which is regulated by ESAT-6, could be a potential molecular mechanism of ESAT-6 induced macrophage apoptosis and may play an important role in the extracellular transmission of Mtb.

Conclusions

In conclusion, the present study demonstrated that treatment with ESAT-6 induces macrophage apoptosis by regulating lncNEAT1/miR-125b-5p/TNF-α pathway. In addition, lncNEAT1 may be involved in facilitating cell death in macrophages by targeting miR-125b-5p/TNF-α axis. Therefore, ESAT-6 or lncNEAT1 may be considered a possible therapeutic target for TB treatment. However, there have some limitations of this work. Firstly, the deeper biological mechanism of ESAT-6 modulates lncNEAT1 expressions still unclear. In this research we only investigated the influence of ESAT-6 on lncNEAT1 expressions level, the direct and indirect relationship between ESAT-6 and lncNEAT1 remains unclear. Secondly, the deeper regulation mechanisms of TNF-α on macrophage apoptosis still unclear in this work. However, some related pathways have been described according to literature, the exact molecular mechanisms of TNF-α on macrophage apoptosis need to be explored in future investigation.

Abbreviations

ESAT-6, Early Secreted Antigenic Target-6; lncNEAT1, Long non-coding RNA nuclear enriched abundant transcript-1; miR-125b-5p, microRNA 125b-5p; Mtb, Mycobacterium tuberculosis; PI, Propidium Iodide; PMA, Phorbol 12-myristate 13-acetate; TB, Tuberculosis; TNF-α, Tumor Necrosis Factor-alpha; UTR, Untranslated Region; RT-qPCR, Reverse-transcription quantitative real-time polymerase chain reaction.

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Disclosure

The authors declare no conflict of interest.

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