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Original Article

LTBP1 inhibits severe pneumonia caused by *Staphylococcus aureus* following cytomegalovirus reactivation via regulation of TGF-β1/Smad signaling pathway

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Abstract

Introduction: Severe viral and bacterial pneumonia are among the most common causes of death worldwide. This study investigated the effects and mechanisms of latent transforming growth factor beta binding protein 1 (LTBP1)'s on methicillin-sensitive *Staphylococcus aureus* (MSSA)-induced severe pneumonia following cytomegalovirus (CMV) reactivation.

Methodology: A young mouse model of severe pneumonia was established using *Staphylococcus aureus* and CMV. LTBP1 overexpression was induced, and pathological changes in lung tissue were assessed through H&E staining. Serum levels of inflammatory factors, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interleukin (IL)-1β, were measured using ELISA. Bacterial load in the lungs was quantified, and protein expression levels of LTBP1, TGF-β1, Smad2, p-Smad2, Smad3, and p-Smad3 in lung tissue were analyzed using Western blot.

Results: The LTBP1 expression was reduced in the young mouse model of severe pneumonia induced by *Staphylococcus aureus* after cytomegalovirus reactivation. Overexpression of LTBP1 inhibited lung damage, reduced serum levels of inflammatory factors (IL-6, IL-1β, and TNF-α), and decreased bacterial load in the lungs. Additionally, overexpression of LTBP1 inhibited the activation of the TGF-β1/Smad signaling pathway.

Conclusions: LTBP1 efficiently reduces severe pneumonia by activating the TGF-β1/Smad signaling pathway, highlighting its potential as a therapeutic target for treating this condition.

Key words: LTBP1; severe pneumonia; methicillin-susceptible *Staphylococcus aureus*; cytomegalovirus; TGF-β1/Smad.

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Introduction

Severe pneumonia is an infectious lung disease caused by pathogenic microbes, with its severity influenced by factors such as viral-bacterial co-infection, bacterial load, and host immunity. Among these, an uncontrolled inflammatory response to pathogen infection plays a pivotal role in disease progression and severity [1]. Globally, pneumonia is one of the leading causes of morbidity and mortality in children under five years of age. Effective treatment of juvenile pneumonia has the potential to reduce pneumonia-related mortality by 32–72% [2,3]. During the early stages of severe pneumonia, excessive production of pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), coupled with a reduction in the anti-inflammatory cytokine interleukin-10 (IL-10), can lead to a "cytokine storm". This can trigger severe pathophysiological events, exacerbating the disease [4]. The increasing misuse of antibiotics and the emergence of drug-resistant microorganisms have become significant issues for intensive care unit physicians [5]. Therefore, early identification of critical therapeutic targets and timely administration of effective interventions are essential to prevent cytokine storms and improve outcomes in severe pneumonia.

Proteomic studies have indicated latent transforming growth factor beta binding protein 1 (LTBP1) as a diagnostic marker for pneumonia and a potential age-related prognostic biomarker in pediatric pneumonia [6]. LTBP1, an extracellular matrix protein, is highly expressed in tissues such as the heart and lung, and is involved in the prevention of numerous illnesses [7]. For instance, LTBP1-mediated T cell suppression has been implicated in the therapeutic effects of human pluripotent stem cell-derived pericyte transplantation for pulpitis [8]. LTBP1 also plays a role in skin regeneration through lactate-induced KAT8-mediated

lactylation at lysine 752 [9]. Additionally, targeting LTBP1 and Protein Phosphatase 2 Catalytic Subunit Alpha has been shown to improve post-myocardial infarction healing during ischaemic preconditioning by inducing serum exosomal miR-133a-3p [10]. Subsequent research has demonstrated that LTBP1 participates in the secretion and activation of TGFβ1 by forming a large potential complex with TGFβ and its propeptide [11]. However, the role of LTBP1 in acute pneumonia, particularly in infants, remains poorly understood.

This study aimed to evaluate the expression of LTBP1 in young mice model of severe pneumonia, as well as to investigate its impact and possible mechanism in *Staphylococcus aureus*-induced severe pneumonia *in-vivo*.

Methodology

Strains

The methicillin-sensitive *Staphylococcus aureus* (MSSA) strain (ATCC 25923; American Type Culture Collection, Manassas, VA), known to cause staphylococcal pneumonia, was obtained and cultivated in Luria Bertani (LB) medium containing 50% LB broth and 50% glycerol at -80˚C. The Smith strain of murine cytomegalovirus (MCMV) (ATCC-VR-1399) was procured from the American Type Culture Collection. MCMV strains were propagated in NIH3T3 cells and titrated using mouse embryonic fibroblasts.

Animal model

Twenty-four pathogen-free female BALB/c mice, aged six weeks and weighing 22–25 g, were obtained from Beijing SPF Biotechnology Co., Ltd. The mice were housed individually in steel micro-isolation cages under a 12-hour light–dark cycle, and fed with water and a regular laboratory diet. The experimental protocols and animal ethics were approved by the Ethics Committee of Changchun University of Chinese Medicine Affiliated Hospital.

The procedures for establishing the animal model were carried out as described previously [12]. Following anesthesia, BALB/c mice were infected intraperitoneally with 3×10^4 PFU MCMV Smith strain or normal saline as a control. To induce viral reactivation, all animals in the MCMV group received cecal ligation and puncture (CLP) four months after the initial MCMV infection. Fourteen days post-CLP, surviving mice in all groups received an intraperitoneal injection of 50 μL containing 5×10^8 CFU of MSSA to induce severe pneumonia.

Experimental designs

The mice were randomly distributed into four groups: control (normal saline + CLP); model (MCMV + CLP + MSSA); model+ad-NC (MCMV + CLP + MSSA + ad-NC); model + ad-LTBP1 (MCMV + CLP + MSSA + ad-LTBP1). Adenoviral vector containing the LTBP1-3flag (Ad-LTBP1) gene was obtained from Shanghai Shibo Medical Biotechnology Co., Ltd., and was administered intratracheally into the mice at a concentration of 1×10^9 PFU seven days prior to the intraperitoneal injection with MCMV Smith strain.

Hematoxylin-Eosin (H&E) staining

Lung tissues were fixed in 4% paraformaldehyde (M13405, Meryer, China) for 24 hours, embedded in paraffin, and sectioned into 5 μm thick slices. These paraffin slices were dewaxed with xylene and then hydrated using a sequence of alcohol solutions: 5 minutes in 100% alcohol, 2 minutes in 95% alcohol, 2 minutes in 80% alcohol, and 2 minutes in 70% alcohol. The hydrated sections were stained using H&E staining (G1120, Solarbio, China) for 15 minutes, followed by differentiation with 0.5% hydrochloric acid alcohol for 30 seconds. Following a 5-minute hematoxylin soak at 50°C, the slices were stained for 40 seconds with eosin. After staining, the sections were cleaned, dehydrated using an alcohol solution, and cleared with xylene. Finally, the stained sections were examined under a light microscope (DM1000LED, Leica, Germany).

Immunohistochemistry

For immunohistochemical detection, lung tissue was embedded in paraffin, fixed with 4% paraformaldehyde for a whole day, and then sectioned into 5 μm thick pieces. The sections were deparaffinized with xylene and hydrated through a graded series of alcohol solutions. To block non-specific binding, the sections were treated with 5% bovine serum albumin (BSA) for 20 min. The sections were then coated with first-generation rabbit anti-LTBP1 polyclonal antibody (1: 100, ab78294, abcom) and incubated overnight at 4°C. The following day, the sections were incubated at room temperature with goat anti-rabbit IgG (1: 800, K0034G-AF594, Solarbio) labeled with horseradish peroxidase. The antibody-antigen complexes were visualized using DAB (SW1020, Solarbio) as a chromogen for 10 minutes, followed by counterstaining with hematoxylin for 2 minutes. Finally, the stained sections were examined under a light microscope at 200× magnification.

ELISA

The levels of several cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, were measured in mouse serum using an ELISA kit following the manufacturer's instructions. The ELX800 UV universal microplate reader was used to read 96-well microplates at 450 nm.

Bacterial counts in lung tissues

To determine bacterial counts in lung tissues, the lungs were crushed and homogenized in 1 mL of phosphate-buffered saline (PBS). The resulting lung homogenates were serially diluted ten times and cultivated on 5% horse blood agar plates (BioMerieux, SA, Marcy-l'Etoile, France). The plates were incubated in anaerobic conditions at 37°C. Colonies were detected following bacterial culture by cultivating on Bertin Pharma's Chapman medium (Montigny le Bretonneux, France).

Western blot

Lung tissues were lysed in cold RIPA buffer (R0010, Solarbio) containing protease and phosphatase inhibitors for 15 minutes in an ice bath, and then centrifuged at 12000g for 25 minutes at 4°C. Total protein was extracted using a protein extraction kit (BC3640-50T, Solarbio). Protein samples (40 μg) were separated on a 10% SDS-PAGE (Bio-Rad Laboratories, Inc., USA), transferred to a PVDF membrane (EMD Millipore, USA), blocked with 5% skimmed milk powder at 4°C for an hour, and then incubated with primary antibody diluted in 5% BSA for overnight at 4°C. The following day, the membrane was incubated with secondary antibody containing goat anti-rabbit IgG (1: 1000, K0034G-AF594, Solarbio) tagged with horseradish peroxidase for one hour at room temperature. Following incubation with secondary antibody, the membrane was washed three times with TBST. Protein bands were visualized using ECL chemiluminescence reagent (GE2301, Genview, China). Protein expression levels were quantified using ImageJ software. The primary antibodies used were: TGF-β1 (ab215715, 1: 1000, abcom), Smad2 (1: 1000, ab40855, abcom), p-Smad2 (1: 1000, ab280888, abcom), Smad3 (1: 1000, ab40854, abcom), p-Smad3 (1: 1000, ab52903, abcom) and GAPDH (ab9485, 1: 1000, abcom).

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical data analysis. Student's *t-test* was used to compare between two groups, and one-way analysis of variance was used to compare several groups. The results were expressed as mean ± standard deviation. A $p < 0.05$ was considered statistically significant.

Results

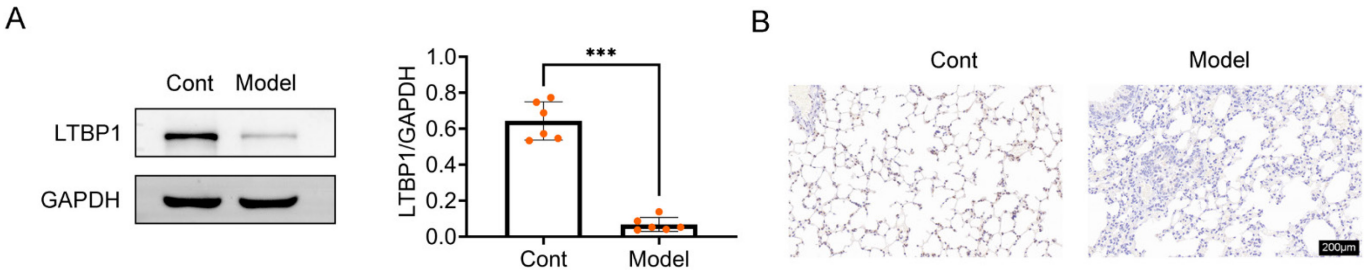
LTBP1 expression is reduced in the severe pneumonia model of young mice

To investigate the potential role of LTBP1 in mitigating severe pneumonia, we assessed LTBP1 expression levels in the lung tissue of young mice in both the control and model groups. Western blot analysis revealed a reduction in LTBP1 expression in the lung tissue of the model group compared to the control group (Figure 1A). Additionally, immunohistochemical staining demonstrated a decrease in the amount of LTBP1 in the lung tissue of young mice in the model group (Figure 1B). These findings suggest that LTBP1 may play a critical role in preventing the onset and progression of severe pneumonia.

Overexpression of LTBP1 inhibits lung injury in young mice with severe pneumonia

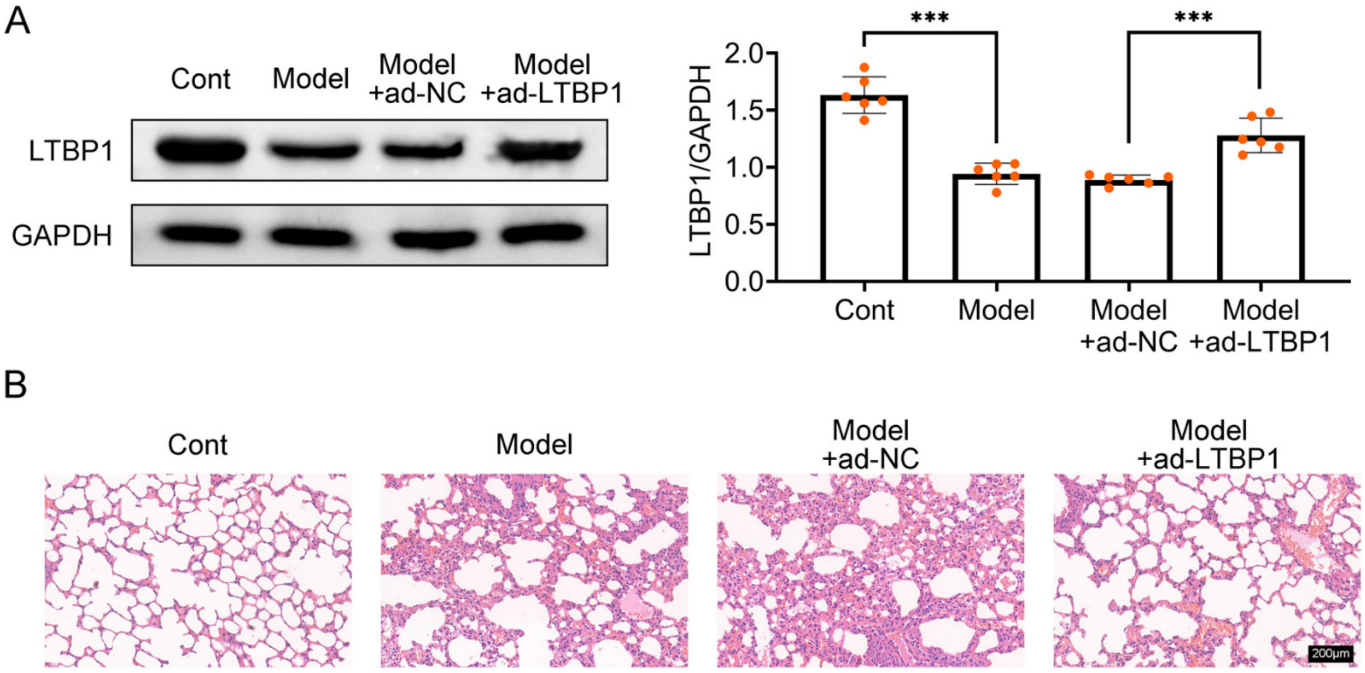
To examine the role of LTBP1 in severe pneumonia, we overexpressed LTBP1 in young mice.

Figure 1. LTBP1 expression is reduced in the severe pneumonia model of young mice.



(A) Protein expression of LTBP1 in lung tissue. (B) Immunohistochemical staining of LTBP1 in lung tissue. Values are presented as mean ± SD. *** $p < 0.001$ versus control group. n = 6.

Figure 2. Overexpression of LTBP1 inhibits lung injury in young mice with severe pneumonia.



(A) Protein expression of LTBP1 in lung tissue. (B) H&E staining to detect pathological changes of lung tissue in each group. Values are presented as mean ± SD. *** $p < 0.001$. n = 6.

The successful overexpression of LTBP1 was confirmed by Western blot (Figure 2A). Histological examination revealed severe pathological changes in the lung tissue of the model group, including alveolar wall thickening, alveolar hemorrhage, and infiltration of inflammatory cells (Figure 2B). In contrast, the lung tissue of the control group showed no obvious histological abnormalities. Remarkably, LTBP1 overexpression therapy led to considerable improvement in the aforementioned histological alterations. This indicated that LTBP1 overexpression has a protective effect against acute lung injury in severe pneumonia.

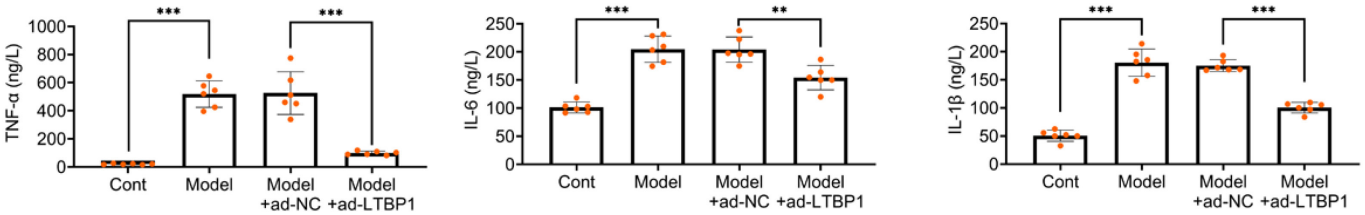
Overexpression of LTBP1 inhibits inflammation in young mice with severe pneumonia

As shown in Figure 3, the levels of inflammatory cytokines TNF-α, IL-1β, and IL-6 were considerably increased in young mice in the model group as compared to the control group. However, overexpression of LTBP1 successfully decreased the synthesis of these inflammatory cytokines. These findings suggest that LTBP1 plays a protective role in severe pneumonia by mitigating inflammatory damage in young mice.

Overexpression of LTBP1 reduces bacterial load in the lungs of young mice with severe pneumonia

As demonstrated in Figure 4, the young mice in the model group exhibited significantly higher bacterial counts in their lungs five days after infection with MSSA pneumonia. However, antibiotic treatment, facilitating overexpression of LTBP1, markedly reduced the bacterial load. These findings imply that LTBP1 overexpression may be involved in enhancing bacterial clearance in severe pneumonia.

Figure 3. Overexpression of LTBP1 inhibits inflammation in young mice with severe pneumonia.



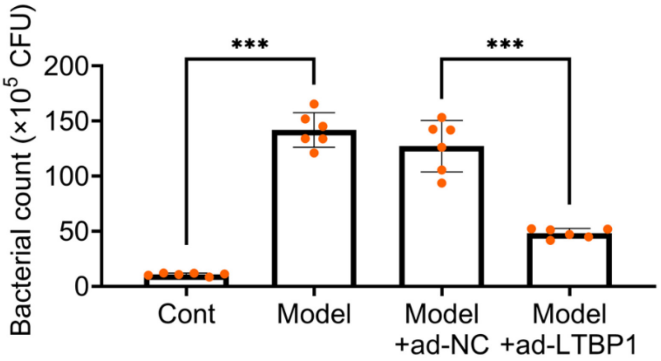
ELISA to detect the levels of inflammatory factors (IL-6, IL-1β, TNF-α) in serum. Values are presented as mean ± SD. ** $p < 0.01$, *** $p < 0.001$. n = 6.

Overexpression of LTBP1 inhibits the TGF-β1/Smad pathway

The expression levels of proteins associated with the TGF-β1/Smad pathway in lung tissue were assessed using Western blotting. In the model group, the levels of p-Smad2/Smad2, p-Smad3/Smad3, and TGF-β1 were considerably increased compared to the control group. However, overexpression of LTBP1 significantly decreased these levels (Figure 5).

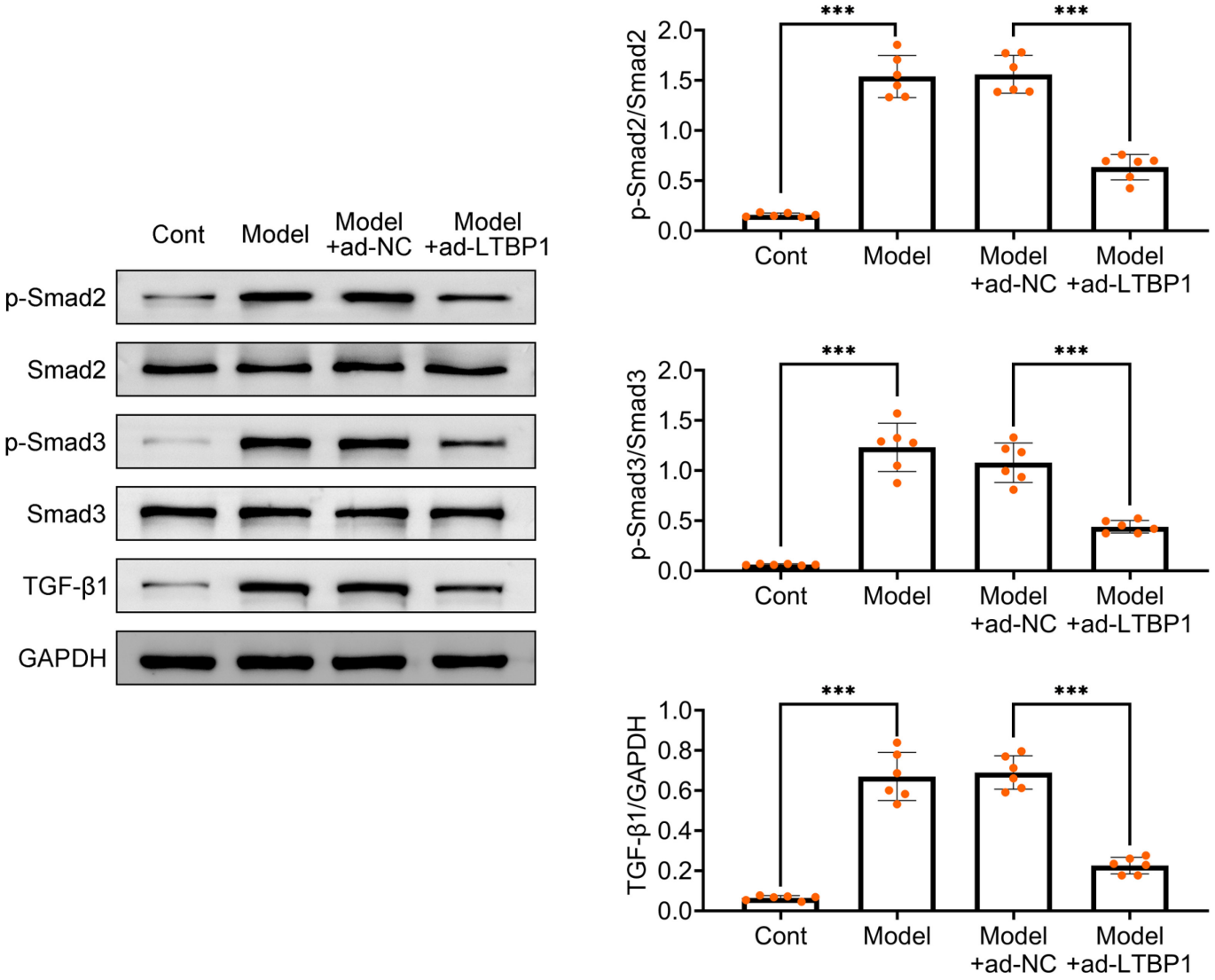
Overall, the findings indicated that LTBP1 may protect against acute lung injury caused by severe pneumonia by promoting bacterial clearance, lowering the inflammatory response, and modulating the TGF-β1/Smad pathway.

Figure 4. Overexpression of LTBP1 reduces bacterial load in the lungs of young mice with severe pneumonia.



Number of bacteria in lung tissue. Values are presented as mean ± SD. *** $p < 0.001$. n = 6.

Figure 5. Overexpression of LTBP1 inhibits the TGF-β1/Smad pathway.



Western blotting to detect the expression of TGF-β1, Smad2, p- Smad2, Smad3, and p-Smad3 proteins. Values are presented as mean ± SD. *** $p < 0.001$. n = 6.

Discussion

Staphylococcus aureus, a common commensal bacterium, makes up between 20% and 30% of the total bacterial population [13]. Under pathogenic conditions, it can cause pneumonia and even death in extreme circumstances [14]. The World Health Organization has classified it as a pathogen that requires immediate attention [15]. The increasing rate of resistance to several drugs due to overuse and abuse of antibiotics has put researchers under pressure to create new, potent tactics to fight bacterial diseases.

The quest for novel antibacterial targets has long plagued researchers. Evidence suggests that therapeutic approaches focusing on inhibiting bacterial virulence factors and modulating host immune responses are more effective than simply eradicating bacteria. These strategies aim to reduce the severity of pneumonia by mitigating the damage caused by the infection [16]. Previous research has demonstrated the importance of LTBP1 targeting in bacterial elimination and virus-induced carcinogenesis [17]. Here, we discovered that LTBP1 regulates lung injury in young mice with pneumonia, and we further confirmed that LTBP1 acts via the TGF-β1/Smad pathway to lessen lung damage in young mice with pneumonia.

Overzealous inflammatory response activation, characterized by inflammatory cell buildup and significant tissue damage, is frequently associated with acute pneumonia [18,19]. Strong anti-inflammatory activity of LTBP1 has also been documented in existing studies. For example, a study has shown that LTBP1 prevents doxorubicin-induced inflammation in cardiomyocytes [20]. Inflammatory markers such as IL-1β, TNF-α, and IL-6 play a critical role in amplifying the inflammatory cascade by inducing chemotactic cytokines [21]. Increased levels of TNF-α, IL-6, and IL-1β may stimulate immunosuppression, which in turn encourages subsequent bacterial infections [22]. The ELISA results of this study show that overexpression of LTBP1 may suppress the expression levels of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in young mice. Additionally, histopathological analysis demonstrated that LTBP1 overexpression can prevent inflammatory cell infiltration and improve the pathological state of lung tissue.

Addressing the global challenge of antibiotic resistance requires innovative approaches, including the development of alternative therapies [23]. Phage therapy has recently emerged as a potential substitute for combating the emergence of antibiotic resistance. Additionally, topical antimicrobial agents have demonstrated excellent clinical efficacy in treating and preventing invasive lung infections, making them a valuable adjunct to antimicrobial therapy in the management of pneumonia [24,25]. In our study, young mice infected with *Staphylococcus aureus* following MCMV exposure exhibited increased lung bacterial counts, indicating impaired bacterial clearance. However, LTBP1 overexpression effectively countered this effect, enhancing bacterial clearance in the lungs.

Studies in the literature have shown that blocking the TGF-β1/Smad signaling pathway can prevent pulmonary fibrosis [26]. Additionally, LTBP1 has been shown to control the TGF-β/Smad system, slowing down the growth of T cell lymphoma and natural killer cells [27]. Upon activation by TGF-β, the TGF-β receptor can phosphorylate members of the Smad family, particularly Smad2 and Smad3, to mediate various biological processes. To investigate the association between LTBP1 and the TGF-β/Smad pathway in more detail, we measured the expression levels of pathway-related proteins in young mice with severe pneumonia using the Western blot. Our findings demonstrated that LTBP1 overexpression can decrease the elevated levels of TGF-β, p-Smad2, and p-Smad3 induced by *Staphylococcus aureus* infection. These results suggest that the TGF-β/Smad pathway plays a critical role in this process and that LTBP1 modulates this pathway to mitigate the effects of severe pneumonia.

Conclusions

Overall, our study demonstrates that LTBP1 plays a protective role in severe pneumonia induced by *Staphylococcus aureus* following CMV reactivation. By inhibiting the TGF-β1/Smad pathway, LTBP1 effectively mitigates lung damage, suppresses inflammation, and enhances bacterial clearance in juvenile mice. These findings provide valuable insights into the potential clinical application of LTBP1 as a therapeutic target for the management of severe pneumonia.

Ethics approval

Ethical approval was obtained from the Ethics Committee of Changchun University of Chinese Medicine Affiliated Hospital (Approval no. 2024760).

Consent to participate statement

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Data availability

All data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon request.

Authors Contributions

Shubo Zhang and Hui Zhang designed the study and carried it out. Shubo Zhang, Hui Zhang, Xiaolin Ju supervised the data collection, Shubo Zhang, Hui Zhang, Xiaolin Ju analyzed the data, Shubo Zhang, Hui Zhang, Xiaolin Ju interpreted the data, Shubo Zhang and Hui Zhang prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Conflict of interests

No conflict of interests is declared.

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