


Tumor necrosis factor- α small interfering RNA alveolar epithelial cell-targeting nanoparticles reduce lung injury in C57BL/6J mice with sepsis

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Abstract

Background: The role of tumor necrosis factor (TNF)- α small interfering (si)RNA alveolar epithelial cell (AEC)-targeting nanoparticles in lung injury is unclear.

Methods: Sixty C57BL/6J mice with sepsis were divided into normal, control, sham, 25 mg/kg, 50 mg/kg, and 100 mg/kg siRNA AEC-targeting nanoparticles groups (n = 10 per group). The wet:dry lung weight ratio, and hematoxylin and eosin staining, western blotting, and enzyme-linked immunosorbent assays for inflammatory factors were conducted to compare differences among groups.

Results: The wet:dry ratio was significantly lower in control and sham groups than other groups. TNF- α siRNA AEC-targeting nanoparticles significantly reduced the number of eosinophils, with significantly lower numbers in the 50 mg/kg group than in 25 mg/kg and 100 mg/kg groups. The nanoparticles also significantly reduced the expression of TNF- α , B-cell lymphoma-2, caspase 3, interleukin (IL)-1 β , and IL-6, with TNF- α expression being significantly lower in the 50 mg/kg group than in 25 mg/kg and 100 mg/kg groups.

Conclusion: TNF- α siRNA AEC-targeting nanoparticles appear to be effective at improving lung injury-related sepsis, and 50 mg/kg may be a preferred dose option for administration.

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Keywords

Tumor necrosis factor- α , siRNA, nanoparticle, lung, sepsis, alveolar epithelial cells

Date received: 20 October 2020; accepted: 4 December 2020

Background

Sepsis is a common, clinically critical disease characterized by high morbidity, serious complications, and high mortality.¹⁻³ Its essence is dysregulation of the host's immune response caused by infection, and it manifests as a clinical syndrome of life-threatening organ dysfunction.⁴ Its mortality is reported to be around 30%, although patients with severe sepsis and septic shock can have a higher mortality of up to 52%.^{5,6} If sepsis is not treated effectively or promptly, it can lead to the development of multiple organ dysfunction syndrome (MODS).⁷ However, existing clinical treatments are limited, and there is an urgent need for novel therapies. Therefore, the development of new drugs and treatments, and the construction of safe, efficient, and targeted treatment systems have become the focus of sepsis-related research.

The pathogenesis of sepsis is not completely clear. Lung injury has been identified in the early stage of disease, and excessive inflammation is recognized as an important cause of MODS.⁸ Therefore, controlling the inflammatory response early on appears to be key. Various bacteria release tumor necrosis factor (TNF)- α or bacterial toxins which stimulate mononuclear macrophages, and activate downstream signaling pathways to further trigger inflammatory reactions.⁹ Therefore, moderate regulation of TNF- α activity and its downstream signal transduction pathways can effectively inhibit the excessive release of inflammatory mediators,¹⁰

which is of great importance in the treatment of sepsis.

RNA interference (RNAi) refers to the phenomenon of knocking out or turning off the expression of specific genes, and is induced by homologous and specific double-stranded RNA. This technology has been widely used to explore gene function and in the field of gene therapy for infectious diseases and malignant tumors. At present, clinical research related to the treatment of sepsis mainly focuses on neutralizing endotoxins, inhibiting the recognition of effector molecules, and injecting anti-inflammatory drugs. Numerous studies^{11,12} have confirmed that RNAi technology has great potential and superiority in gene therapy of organ damage. Additionally, it was reported that the intraperitoneal injection of TNF α -small interfering (si)RNA liposomes attenuated liver injury in a model of acute sepsis induced by lipopolysaccharide (LPS).¹³ Therefore, RNAi technology could be used to inhibit the expression of TNF- α in lung tissue to avoid lung injury caused by excessive inflammation.

Poly(lactic acid-glycolic acid) (PLGA) copolymer is a functional polymer randomly polymerized from lactic acid and glycolic acid. It is readily synthesized, has a stable quality, good biocompatibility, and is degradable, non-toxic, non-irritating, non-immunogenic, and can be used for sustained drug release.¹⁴ PLGA has been certified by the US Food and Drug Administration and approved for use in tissue engineering, medical materials, and drug delivery. To achieve the targeted

release of nanoparticles, it is particularly important to determine the specific physical, chemical, or biological characteristics of the intended lesion. Dipalmitoyl lecithin is mainly distributed in the lung alveolar epithelium and is an ideal focus for the targeted release of nanoparticles.¹⁵ Therefore, we attempted to use RNAi technology to silence the characteristics of specific gene expression by constructing PLGA-mediated TNF- α -siRNA alveolar epithelial cell (AEC)-targeting nanoparticles. These were administered to C57BL/6J mice with sepsis as a novel approach to gene- and drug-targeted therapy.

Methods

Ethical considerations

Animal experiments were certified and approved by the Animal Care and Use Committee of our hospital (AC201800987-2c), and complied with the guidelines of Animal Research: Reporting of In Vivo Experiments (ARRIVE). Adequate care was taken for the benefits of animals

according to the ‘Guide for the Care and Use of Laboratory Animals, 8th Edition’,¹⁶ and efforts were made to minimize the number of animals used and to decrease their suffering.

Study design

Sixty C57BL/6J mice were purchased from Suzhou Laihui Biotechnology Co., Ltd. (Suzhou, China). Mice were divided into the following groups: normal, control, sham, 25 mg/kg, 50 mg/kg, and 100 mg/kg (n = 10 per group; Figure 1). The normal group received no intervention, the control group received LPS (10 mg/kg) instillation into the trachea to establish the acute endotoxin lung injury model,¹⁷ and the sham group received a saline injection in the tail vein, and LPS (10 mg/kg) instillation into the trachea 24 hours later. For the other three groups, 25 mg/kg, 50 mg/kg, or 100 mg/kg TNF- α -siRNA AEC-targeting nanoparticles were injected, and LPS (10 mg/kg) was instilled into the trachea 24 hours later. Two days after the procedure, the mice were sacrificed by cervical dislocation, and the lung tissue and bronchoalveolar fluid

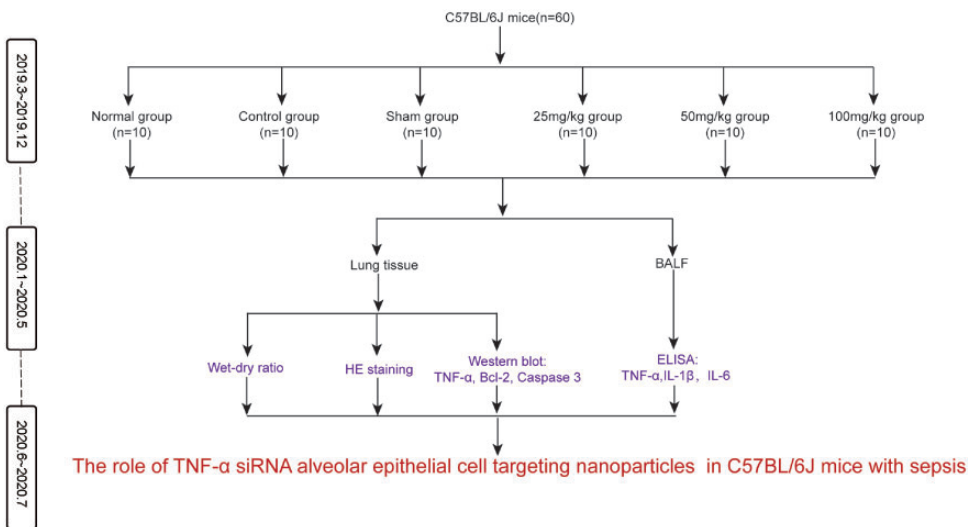


Figure 1. Flow chart of the study design.

were collected and analyzed. Then, the wet: dry lung weight ratio, hematoxylin and eosin (HE) staining, western blotting, and enzyme-linked immunosorbent assay (ELISA) of inflammatory factors were conducted to investigate the role of TNF- α siRNA AEC-targeting nanoparticles in C57BL/6J mice with sepsis.

TNF- α siRNA AEC-targeting nanoparticle preparation

The double emulsion solvent evaporation method was used to construct TNF- α siRNA AEC-targeting nanoparticles. We dissolved TNF- α siRNA (Heshen, Shanghai, China) in diethylpyrocarbonate-treated water at a concentration of 200 μ M to form an internal aqueous phase solution. Then alveolar surfactant-Ab (35 mg/ml) dissolved in ethyl acetate was accurately weighed and used to prepare a solution as the oil phase. We added 1 mL solution of internal aqueous phase to 12 mL of oil phase under ultrasonic conditions of 150 W 30 s to form a milky white water/oil (W1/O) colostrum solution. Then we added the colostrum solution to 10 mL of external aqueous phase solution (2% polyvinyl alcohol) under homogeneous emulsification (322.5 \times g, 60 s) to make the W1/O W2 double emulsion. We stirred this emulsion at low speed for 4 hours to completely evaporate the organic solvent and to obtain solidified nanoparticles. Finally, the nanoparticles were collected by centrifugation at 645 \times g for 30 minutes in a high-speed centrifuge, then were resuspended and washed three times with deionized water, and freeze-dried for 24 hours to obtain nanoparticles coated with TNF- α -siRNA. The nanoparticles were further evaluated as previously reported.^{18,19} We used the Zetasizer 3000HS particle size and surface potential analyzer (Hengsheng Biotechnology Co., Wuxi, China) to determine the size and surface potential of the nanoparticles. These were

shown to be round and approximately 250 nm in diameter.

Measurement of wet:dry lung weight ratio

We removed the lungs from sacrificed mice, rinsed their surface with normal saline, dried surface liquid with filter paper, then weighed the tissue which was defined as the wet weight. The lungs were then dried in the oven at 80°C overnight and weighed again to obtain the dry weight. The wet/dry weight calculation was used as the ratio.

HE staining

Dehydrated lung tissues were embedded in paraffin and the transverse section of collected tissues was discreetly sliced for HE staining as previously reported.²⁰ Specimens were then photographed and analyzed under a microscope (Olympus X100, Tokyo, Japan) at a magnification of \times 400. For data analysis, we calculated and compared the number of eosinophils in up to five fields of view per group.

Western blotting and ELISA

Frozen lung tissue samples were lysed mechanically in cell lysis buffer for total protein extraction using a protein extraction kit (Abcam, Cambridge, MA, USA). Lysates were centrifuged at 275 \times g for 15 minutes at 4°C, and the concentration was measured by the bicinchoninic acid method. Samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electro-transferred onto nitrocellulose membranes. Membranes were then blocked with 5% non-fat milk for 1 hour at room temperature, then incubated with rabbit anti-TNF- α , rabbit anti-caspase 3, or rabbit anti-B-cell lymphoma (Bcl)-2 primary antibodies (all Abcam) for 2 hours at 4°C. An anti- β -tubulin primary antibody (Abcam) was used as the loading control. The membranes were washed three times

for 5 minutes each in Tris-buffered saline + 0.1% Tween 20, then incubated with anti-goat secondary antibody (Abcam) for 2 hours at room temperature. Protein bands were visualized using enhanced chemiluminescence, and the relative expression was evaluated with ImageJ software.

ELISA was carried out for TNF- α , interleukin (IL)-1 β , and IL-6 using commercially available kits (Abcam) according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times to confirm consistent results. SPSS 23.0 software (SPSS, Chicago, IL, USA) was used for data analysis. Analyzed data are expressed as the mean \pm SEM. Differences between two groups were compared using the Student's *t*-test, and $p < 0.05$ was considered statistically significant.

Results

Verification of TNF- α siRNA AEC-targeting nanoparticles

As shown in Figure 2, TNF- α gene expression was significantly inhibited by TNF- α

siRNA AEC-targeting nanoparticles, indicating that their construction was successful.

The wet:dry lung weight ratio

The wet:dry lung weight ratio in control and sham groups was significantly lower than in normal, 25 mg/kg, 50 mg/kg, and 100 mg/kg groups (all $p < 0.05$; Figure 3).

HE staining and eosinophil number

In control and sham groups, the partial collapse of alveoli was evident, together with

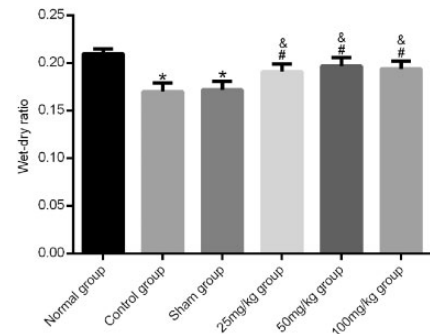


Figure 3. Wet:dry lung weight ratio among groups. * $p < 0.05$ compared with the normal group; # $p < 0.05$ compared with the control group; & $p < 0.05$ compared with the sham group.

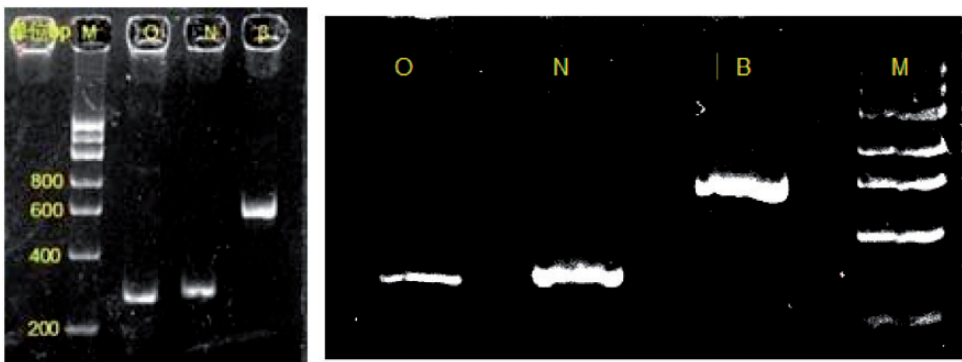


Figure 2. Gene expression in AECs before and after TNF- α siRNA intervention. Left: control specimens; right: experimental specimens.

AEC, alveolar epithelial cell; TNF, tumor necrosis factor; O, oat dodine agar; N, number of loading cycles; B, bacillus.

the thickening and destruction of alveolar septa and alveolar interstitial edema (Figure 4). By contrast, interstitial structures were intact, and there was reduced alveolar congestion and infiltration of inflammatory cells in alveolar spaces, and less injury to lung tissue in groups receiving TNF- α siRNA AEC-targeting nanoparticles. Additionally, a significantly higher number of eosinophils was detected in control and sham groups compared with other groups (all $p < 0.001$), suggesting that they were reduced by TNF- α siRNA AEC-targeting nanoparticles. The number of eosinophils in the 50 mg/kg group was significantly lower than in 25 mg/kg and 100 mg/kg groups ($p < 0.05$).

Western blot analysis of TNF- α , Bcl-2, and caspase 3 expression

As shown in Figure 5, the protein expression of TNF- α , Bcl-2, and caspase 3 was significantly higher in control and sham groups than in other groups (all $p < 0.05$), with TNF- α siRNA AEC-targeting nanoparticles significantly reducing their expression. Moreover, TNF- α expression was significantly lower in the 50 mg/kg group than in 25 mg/kg and 100 mg/kg groups (all $p < 0.05$).

ELISA analysis of TNF- α , IL-1 β , and IL-6 expression

TNF- α , IL-1 β , and IL-6 levels in control and sham groups were shown to be

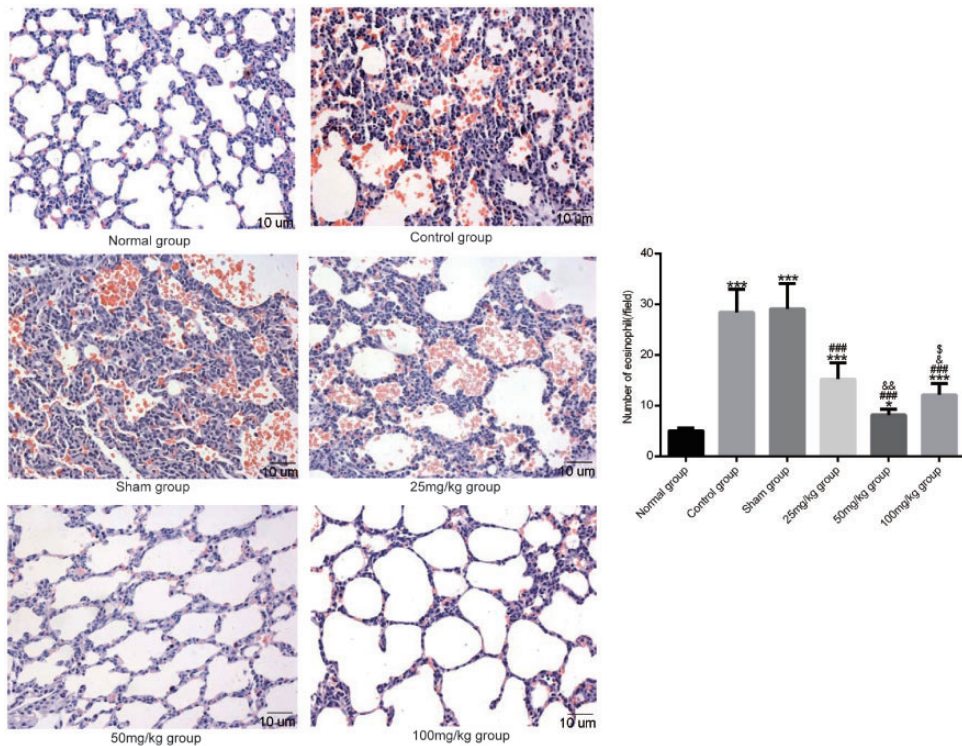


Figure 4. HE staining among groups. * $p < 0.05$, *** $p < 0.001$ compared with normal group; #### $p < 0.001$ compared with sham group; & $p < 0.05$, && $p < 0.01$ compared with 25 mg/kg group; \$ $p < 0.05$ compared with 50 mg/kg group.

HE, hematoxylin and eosin.

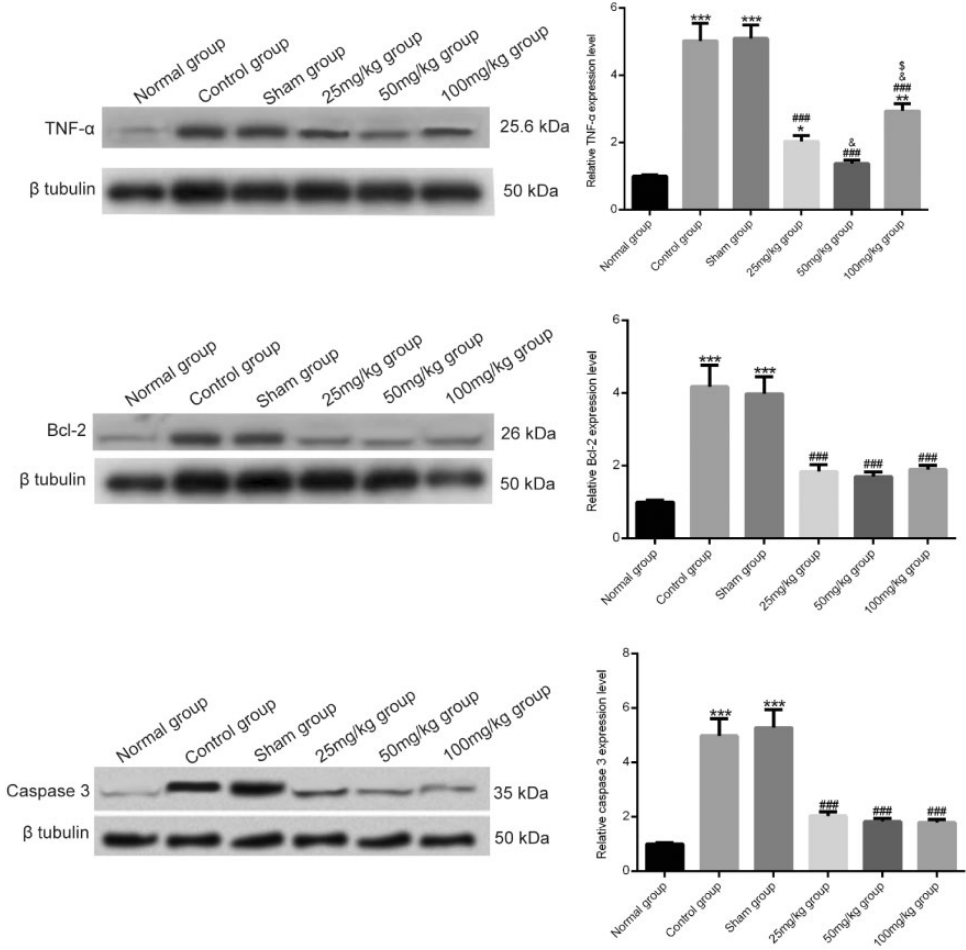


Figure 5. Western blot analysis of TNF- α , Bcl-2, and caspase 3 expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with normal group; ###, $p < 0.001$ compared with sham group; &, $p < 0.05$ compared with 25 mg/kg group; \$, $p < 0.05$ compared with 50 mg/kg group. TNF, tumor necrosis factor; Bcl, B-cell lymphoma.

significantly higher than in other groups by ELISA analysis (all $p < 0.05$; Figure 6).

Discussion

Sepsis is a systemic inflammatory response syndrome caused by infectious factors, and is a widespread complication after severe trauma or burn injuries, and major surgical operations.²¹ MODS is a common cause of death in clinically critical patients with

sepsis, and lung injury is the most common complication in patients with sepsis.²² Therefore, the means of controlling lung injury is of great importance in improving the physical condition and prognosis of such patients.²³ TNF- α plays a key role in excessive inflammation and is a potential target for the treatment of sepsis.²⁴ Our study indicates that TNF- α siRNA AEC-targeting nanoparticles can significantly inhibit the level of TNF- α ,

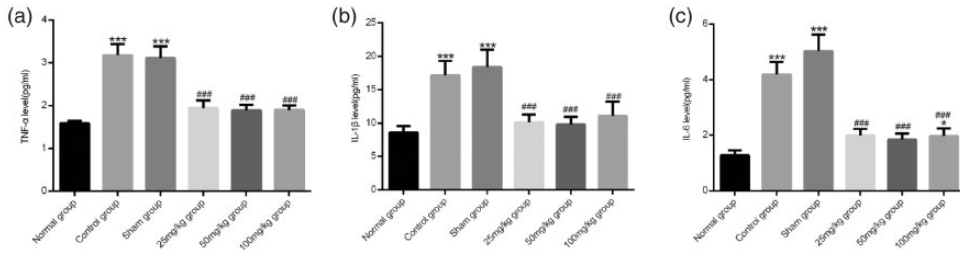


Figure 6. ELISA analysis of TNF- α , IL-1 β , and IL-6 expression. * $p < 0.05$, *** $p < 0.001$ compared with normal group; ###, $p < 0.001$ compared with sham group.

ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor; IL, interleukin.

Bcl-2, caspase 3, IL-1 β , and IL-6 expression, thereby reducing the inflammatory response and improving lung function. Moreover, 50 mg/kg TNF- α siRNA AEC-targeting nanoparticles appears to be the optimal dose for the treatment of lung injury associated with sepsis.

TNF- α is involved in the regulation of various biological phenomena, including apoptosis, cell proliferation, the stress response, innate immune response, and septic shock.²⁵ It controls the promoters of nearly 200 genes stimulated by LPS and inflammatory factors, and its downstream genes such as IL-1 β and IL-6 are involved in the development of sepsis.²⁶ Several studies^{27–29} have reported increased TNF- α activity in sepsis models and the lung tissues of patients with sepsis. This activity is related to the severity of lung injury, so inhibiting TNF- α expression effectively inhibits the downstream signaling pathway, thus reducing lung injury and improving the survival rate.³⁰ Inhibiting TNF- α expression has become a new focus in the treatment of sepsis, but how to do so remains a challenge for healthcare providers.

Composite nanomaterials can make the targeted release of biological agents a reality, and the use of dipalmitoyl lecithin is an ideal method for nanoparticles to target AECs.^{31–33} Exogenous siRNAs must pass through the cell membrane and reach a sufficient concentration in the cytoplasm to

successfully silence the expression of specific genes.³⁴ However, a safe and effective transport vector has become an important obstacle in the development of this.³⁵ Nanoparticles, as a new type of non-viral gene carrier, offer key advantages in gene delivery. Not only do they prevent the rapid degradation of siRNA, but controlling their particle size, and connecting ligands, antibodies, and enzymes can enable active targeting.^{36,37} They are usually in the range of 10 to 100 nm in size, which is ideal as a drug-loading system for intravenous injection.^{38,39} Drugs can be dissolved or encapsulated in nanoparticles, and they are particularly suitable for containing fat-soluble drugs such as statins.⁴⁰

Endotoxins and other stimulating inflammatory cells produce large amounts of inflammatory mediators and lipid metabolites, which promote the recruitment and activation of inflammatory response cells, especially neutrophils and macrophages in the lung tissue. This expands the inflammatory response, leading to a waterfall-like cascade,⁴¹ with resultant osmotic pulmonary edema, destruction of the gas–blood barrier, the formation of transparent membranes, alveolar collapse, and even pulmonary fibrosis.⁴² Large numbers of lung parenchymal cells such as pulmonary microvascular endothelial cells and AECs are not only target cells for inflammatory

cells and inflammatory mediators, but also directly activate inflammatory cells.^{43,44} Therefore, by establishing a model of acute lung injury related to sepsis, we can study underlying pathophysiological mechanisms and explore new forms of treatment.

Several limitations must be considered in this study. First, we only conducted *in vitro* experiments to explore the role of TNF- α siRNA AEC-targeting nanoparticles in C57BL/6J mice with sepsis, so further verification *in vivo* is warranted. Furthermore, we only investigated the effects of 25, 50, and 100 mg/kg TNF- α siRNA AEC-targeting nanoparticles; therefore other concentration of nanoparticles should be tested to determine the optimal dose.

Conclusions

We found that TNF- α siRNA AEC-targeting nanoparticles are effective in the treatment of lung injury in C57BL/6J mice with sepsis by inhibiting the inflammatory response and improving alveolar permeability. While TNF- α siRNA AEC-targeting nanoparticles may be an important approach for the treatment of lung injury related to sepsis, the preparation of nanoparticles requires further refinement. Passive targeting is inefficient, so the preparation of antibody-targeted active nanoparticles should be pursued.


Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This study has been found by Youth Science and Technology Project of Zhangjiagang City Health Commission (ZJGQNKJ201816).

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