The therapeutic effect of a novel anti-TNF- α /IL-6R triple-specific fusion protein under experimental septic condition

Xiaole Chen

Fujian Medical University Union Hospital

Shuangyu Tan

Fujian Medical University

Mengru Yan (✉ 614264592@qq.com)

Fujian Medical University

Kaimei Nie

Fujian Medical University

Qingmei Zheng

Fujian Medical University

Yaduan Wang

Fujian Medical University

Rui Liu

Fujian Medical University

He Wang

Fujian University of Traditional Chinese Medicine

Juhua Yang

Fujian Medical University

Nanwen Zhang

Fujian Medical University <https://orcid.org/0000-0002-6711-1560>

Research Article

Keywords: sepsis, triple-specific fusion protein, cytokine, inflammatory, single domain chain

Posted Date: March 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-298423/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

A novel anti-TNF- α /IL-6R triple-specific fusion protein, by linking 3 single domain chains, was designed and constructed in our lab. The high purity fusion proteins were obtained by our developed prokaryotic expression system process with high binding affinity with TNF- α (94.75 pM), Human Serum Albumin (1.83 nM) and IL-6R (2.29 nM). In this study, the anti-TNF- α /IL-6R triple-specific fusion protein protected the mouse fibroblast fibrosarcoma cell line (L929) from the apoptosis effects induced by TNF- α , establishing that the expressed fusion proteins can selectively combine with TNF- α *in vitro*. *In vivo*, the survival rate of cecal ligation and puncture (CLP) was notably increased in the group with anti-TNF- α /IL-6R triple-specific fusion protein treatment, and meaningfully higher compared with the single-targeted IL-6R and TNF- α fusion protein at the same dose. After the treatment with anti-TNF- α /IL-6R triple-specific fusion protein, the level of serum TNF- α , IL-1 β and IL-6 were significantly decreased, and sepsis-induced pathological injuries in the kidney were remarkably attenuated. The anti-TNF- α /IL-6R triple-specific fusion protein can be the potential candidate for the development of new drug design against sepsis.

1 Introduction

Sepsis constitutes a major healthcare problem which is expected to increase due to an ageing population. In 2017, WHO recognized that sepsis was a global health priority[1]. Sepsis is one of the most severe disease caused by a dysregulated host response to infection [2–3]. Recent studies have demonstrated that the extensive release of cytokines and other mediators play critical roles in the development of sepsis [4]. Tumor necrosis factor alpha (TNF- α) is the closest mediator of the cytokine cascade. Serum TNF- α levels increase within 1–2 hours after injection of endotoxin that may be involved in sepsis. [1, 5, 6]. The antibody or molecules against TNF- α have been developed to treat sepsis, though the clinical trial results were not ideal.

Interleukin-6 (IL-6) is produced as a prominent activator of the acute phase response with extensive biological activities [7, 8]. The plasma half-life of IL-6 is longer than other inflammatory factors such as TNF- α or IL-1 β [9], when the level of TNF- α in the plasma of patients with sepsis is significantly decreased, the concentration of IL-6 is significantly increased [10]. A link between high IL-6 level and mortality in septic patients [6] has been found in the previous studies. Therefore, IL-6, widely accepted as a valuable biomarker to evaluate the therapeutic response and the prognosis of sepsis [5], can be employed as potential therapeutic target in the clinical treatment of sepsis.

Hereby, a triple-specific fusion protein, composed of anti-TNF- α , anti-Human Serum Albumin (HSA) and anti-IL-6R single domain chains, was designed and constructed by us. Different from other conventional single-chain Fv (ScFv) based antibodies from human IgGs, the single domain chain derived from the natural camel heavy-chain only antibodies (HCAbs) [11, 12] presents various superior advantage features, such as lower molecular weight, increased stability, and better solubility [12, 13]. Furthermore, a recombinant fusion protein can be easily constructed by different single domain chains allowing for target one or two antigens at the same time. The novel anti-TNF- α /HAS/IL-6R fusion protein are

supposed to bind to TNF- α and IL-6 receptor, respectively, and also bind to human serum albumin (HSA) leading to significantly increase the half-life of fusion protein in vivo as well as block cytokines at particular anatomical sites [14]. The present study aimed to explore the potential associations of the functions of TNF- α and IL-6 being blocked under experimental septic condition at the same time by a novel anti-TNF- α /IL-6R triple-specific fusion protein, and investigate its therapeutic effects in vitro and vivo.

2 Material And Methods

2.1 The preparation of anti-TNF- α /IL-6R triple-specific fusion protein

The 100 ng plasmid of pET28a-anti-TNF- α /IL-6R designed and constructed by our lab was transformed into competent cell, *E. Coli.BL21(DE3)*, (Beijing Quanshijin Biotechnology Company) by heat shock method. The recombinant cells from 50 ml culture were harvested by centrifugation at 10,000 g for 10 min and the pellet was resuspended in 5mL TGE buffer (50mM Tris pH 7.9, 0.5 mM EDTA, 50 mM NaCl, 5% glycerin), then ultrasonic crushed on the ice for 30min. The fusion protein fused with His- tag were expressed and purified using a BeaverBeads™ IDA-Nickel (Suzhou Beaver Bioengineering Company) and desalted with Äkta (GE Healthcare, USA). The purified fusion protein was analyzed by 10% SDS-PAGE.

2.2 Anti-TNF- α /IL-6R triple-specific fusion protein specificity analysis

The plates were first coated with IL-6R ($1 \mu\text{g}\cdot\text{mL}^{-1}$), TNF- α ($1 \mu\text{g}\cdot\text{mL}^{-1}$) and HSA (Human serum albumin, $1 \mu\text{g}\cdot\text{mL}^{-1}$) respectively at 4°C, overnight (diluted in 0.1 M $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer, pH 9.6). The next day, the plates were washed 3 times with PBST (0.05% Tween-20 in PBS) and blocked for 2 h with 100 μL 1% BSA-PBS. The fusion protein diluent was added at various final concentrations after 3 times washes and incubated for 1 h at 37°C. 50 μL anti-rabbit IgG with His-tag antibody (CST, USA) was added and incubated for another hour. The plates were washed 3 times and then 50 μL goat anti-rabbit IgG-HRP antibody (CST) was added and incubated for another hour. After washing, a total of 100 μL reaction buffer (5 mg o-Phenylenediamine, 10 μL H_2O_2 , 5 mL Citric acid- Na_2HPO_4) was added into the wells. The reaction was stopped by 2 M H_2SO_4 after 10minutes. Absorbance was measured at 492 nm on an ELISA plate reader (Bio Tek, USA).

2.3 Cell-based functional assays of anti-TNF- α /IL-6R triple-specific fusion protein in vitro

Murine L929 fibroblast cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in 1640 (Gibco, USA) supplemented with 10% FBS (Gibco, USA). Approximately 12,000 cells were seeded in each well of 96-well plate and incubated for 24 h at 37°C under a humidified atmosphere and 5% CO_2 . The reaction mixtures were prepared using TNF- α (0.15 nM) and various concentrations of

purified anti-TNF- α /IL-6R triple-specific fusion protein (ranging from 0.032 to 32 nM) in the presence of actionomycin ($1 \text{ mg} \cdot \text{mL}^{-1}$). The mixtures were incubated on ice for 10 min and then added onto the previously L929 seeded wells for 12 h treatment. 20 μL of MTT (5 mg/ml) was added to each well and the plate was kept in the incubator for further 4 h. The supernatant was discarded and 150 μL of dimethyl sulfoxide was added onto wells with incubation for 40 min at room temperature with agitation. The absorbance was measured at 570 nm and the achieved results were fit into dose-response inhibition curve in Prism program (version 5.01, GraphPad Software Inc.).

For apoptotic cells analysis, L929 cells was treated with the same methods mentioned above. After incubation for 12 h, the supernatant was discarded. The plates were washed with PBS. 100 μL of PBS and 5 μL of PI (Propidium Iodide) was added in each well and incubated for 45 min at 37°C. The L929 cells were fixed in 4% paraformaldehyde for 15min. The plates were washed and 100 μL of PBS and 5 μL of DAPI(4',6-diamidino- 2-phenylindol) were added for each wells at room temperature for 30 min. After staining, the cells were imaged and analyzed using an Operetta™ High Content Screening instrument with Harmony™ software version 3.5.2 (PerkinElmer, Waltham, MA, USA) and different excitation/emission channels.

2.4 The therapeutic effect of anti-TNF- α /IL-6R triple-specific fusion protein in a vivo model of animal sepsis

Specific pathogen free (SPF) male Sprague-Dawley rats, weighing 180–200 g, were supplied by Fujian Medical University Animal Center (Fuzhou, China). The rats were housed in Fujian Medical University Animal Center, with free access to food and water, under a light/dark (12 h/12 h cycle) at a temperature of 20–23°C. The rats were acclimatized for more than 7 days before the experiments.

Rats were anesthetized with 2 % pentobarbital sodium ($40 \text{ mg} \cdot \text{kg}^{-1}$, i.p.) and received a laparotomy in the middle of the abdomen. The cecum was isolated, ligated using a 3 – 0 silk and punctured with an 18-gauge needle for two times. The fecal material was expelled by a gently squeezing. In sham-operated rats, the cecum was isolated, but neither ligated nor punctured. The cecum was returned, and the wound was then closed. After surgery, 3 ml/100 g prewarmed saline was given intraperitoneally for fluid resuscitation. Rats were divide into 7 groups ($n = 8$): (1) sham group, (2) CLP model group, (3) low dose of anti-TNF- α /IL-6R triple-specific fusion protein group (CLP + anti-TNF- α /IL-6R $1 \text{ mg} \cdot \text{kg}^{-1}$), (4) middle dose of anti-TNF- α /IL-6R triple-specific fusion protein group (CLP + anti-TNF- α /IL-6R $5 \text{ mg} \cdot \text{kg}^{-1}$), (5) high dose of anti-TNF- α /IL-6R triple-specific fusion protein group (CLP + anti-TNF- α /IL-6R $10 \text{ mg} \cdot \text{kg}^{-1}$), (6) anti-TNF- α fusion protein $5 \text{ mg} \cdot \text{kg}^{-1}$ group, (7) anti-IL-6R fusion protein $5 \text{ mg} \cdot \text{kg}^{-1}$ group. The drugs were intravenously injected half an hour later after operation. Meanwhile, the sham group and the CLP model group were given the same saline intravenously. 72 hours after surgery, rats were scarified, and the blood was collected. Left kidneys were harvested and fixed in 4% paraformaldehyde overnight. Right kidneys were harvested and frozen in liquid nitrogen for molecular biological examinations.

2.4.1 Survival analysis and blood urea nitrogen (BUN) examinations

For Survival analysis, after surgery, the survival status of each group of rats was observed every 12 hours and recorded. The level of creatinine and BUN were examined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.4.2 Hematoxylin-eosin (HE) staining

Kidney tissues were rinsed with ice-cold saline solution and fixed with paraformaldehyde (4 %), embedded in paraffin, and cut into 5 mm sections. Then all sections were stained with HE and photographed under an optical microscope with the magnification of 20×.

2.4.3 Enzyme-linked immunosorbent assays (ELISA)

The levels of TNF- α , IL-6 and IL-1 β were detected with commercial kits following the manufacturer's instructions (ELISA kits of TNF- α , IL-6 and IL-1 β from Shanghai enzyme linked Biotechnology Co., Ltd, Shanghai, China).

2.4.4 Quantification of inflammatory mediator mRNA levels by Realtime PCR

Total mRNA was isolated using an RNA simple Total RNA Kit (Tianmo Biotech, Beijing, China). The concentration of mRNA was determined using a NanoDropND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Single-stranded cDNA was synthesized using oligonucleotide primer and super M-MLV (Takara, Japan) in a 20 μ L reaction system. Quantitative real-time PCR was performed on 1 μ L cDNA using the 2 $\times$ TB Green Premix Ex Taq II (TliRNaseH Plus) (Takara) in a 20 μ L reaction system in a PCR System (Exicycle 96; Bioneer, Taejeon, Korea). Relative gene expression was normalized with GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. The following primers were used:

IL-1 β primer sequences were forward 5'-TGGAGAAGCTGTGGCAGCTACCT - 3' and reverse 5' - GAACGTCACACACCAGCAGGTT-3'

IL-6 primer sequences were forward 5'-GCCAGAGTCCTTCAGAGAGA - 3' and reverse 5' - GGTCTTGGTCCTTAGCCACT-3'

TNF- α primer sequences were forward 5'-AGTGACAAGCCTGTAGCCCACGT-3' and reverse 5'-CCATCGGCTGGCACCAGTAGTT-3'

GAPDH primer sequences were forward 5'-GTGAAGGTCGGTGTGAACG-3' and reverse 5'-CTCGCTCCTGGAAGATGGTG-3'

2.4.5 Statistical analysis

All data are expressed as means $\pm$ SD. One-way ANOVA followed by Bonferroni's Multiple Comparison Test using GraphPad software was used for comparisons among experiment groups. A p-value of less than 0.05 was considered statistically significant.

3 Results

3.1 The preparation of anti-TNF- α /IL-6R triple-specific fusion protein

The cDNA encoding the recombinant anti-TNF- α /IL-6R triple-specific fusion protein, was made synthetically based on the sequence information of patent US2010/0172894 and US2012/0093839. The 3D structure of an anti-TNF- α /IL-6R triple-specific fusion protein was mimicked by SWISS-MODEL (Fig. 1a). The 39.27kb inserts were ligated into the multiple cloning sites region downstream of the pET28a + vector using the EcoRI/NotI restriction sites. Colony PCR results showed that the size of the amplified gene fragment was 1600 bp (Fig. 1b), where the plasmid of anti-TNF- α /IL-6R was transformed into competent cells *BL21(DE3)* successfully. The fusion protein was expressed and analyzed by 10 % SDS-PAGE showed in Fig. 1c, indicating that anti-TNF- α /IL-6R triple-specific fusion protein is expressed in *E.coli* classical inclusion bodies. The inclusion bodies were purified by BeaverBeads™ IDA-Nickel (Fig. 1d) and desalted with Äkta (Fig. 1e). The purity of anti-TNF- α /IL-6R triple-specific fusion protein reached 95%.

3.2 Binding capacity analysis of anti-TNF- α /IL-6R triple-specific fusion protein

As shown in Fig. 1f, anti-TNF- α /IL-6R triple-specific fusion proteins were able to bind TNF- α , HSA and IL-6R in a dose-dependent manner. The results indicate that the EC₅₀ value of anti-TNF- α /IL-6R triple-specific fusion protein was 94.75 pM for TNF- α , 1.83 nM for HSA and 2.29 nM for IL-6R.

3.3 Inhibition of TNF- α by anti-TNF- α /IL-6R triple-specific fusion protein in vitro

The MTT results (Fig. 2A) presented that anti-TNF- α /IL-6R triple-specific fusion protein can inhibit TNF-cytotoxicity on L929 cells in a dose-dependent manner with IC₅₀ values of 19.6 pM. DAPI/PI staining was employed to observe the effect of anti-TNF- α /IL-6R trispecific fusion protein on the apoptosis of L929 cells induced by TNF- α . Under the fluorescence microscope, the negative control group showed uniform blue fluorescence, and the nuclei treated with TNF- α were evenly colored red by PI (Fig. 2B). The results of cell death by High-content analysis exhibited that the control group had an apoptotic rate of (3.40 $\pm$ 0.77) %, which was significantly different from (79.45 $\pm$ 5.89) % of the TNF- α (2 ng/ml) group (P < 0.001). The apoptotic rates of the anti-TNF- α /IL-6R triple-specific fusion protein administration group (32 nM, 3.2 nM, 0.32 nM) were (8.66 $\pm$ 4.93) %, (8.93 $\pm$ 2.16) % and (64.89 $\pm$ 4.69) %, respectively. These results indicate that TNF- α can induce apoptosis of L929 cells, whereas the anti-TNF- α /IL-6R triple-specific fusion protein

administration group can effectively reduce the apoptosis rate of TNF- α -induced apoptosis of L929 cells in a dose-dependent manner (Fig. 2C).

3.4 Anti-TNF- α /IL-6R triple-specific fusion protein significantly alleviates CLP induced sepsis

As demonstrated in Fig. 3a, the survival rate in the sham was 100%, while survival rate of CLP decreased to 10% within 72 h. Different doses of anti-TNF- α /IL-6R triple-specific fusion protein intervention group improved the survival rate of rats within 72 h. The survival rate was 60% in 5 mg·kg⁻¹ group and 30% in 1mg·kg⁻¹ group, where 10mg·kg⁻¹ group was the most significant with the 70% survival rate. Moreover, compared with the single-targeted IL-6R or TNF- α fusion protein, constructed and obtained by our lab as well, anti-TNF- α /IL-6R triple-specific fusion protein displayed the higher survival rate at the same dose.

As shown in the Fig. 3b, compared with the sham group, the expression level of serum urea nitrogen (BUN) in the CLP group increased significantly ($P < 0.001$). The expression levels of BUN were meaningfully decrease in the single-targeted IL-6R or TNF- α fusion protein, and anti-TNF- α /IL-6R triple-specific fusion protein treated groups ($P < 0.05$, $P < 0.01$ or $P < 0.001$), compared with the CLP group. The same dose of anti-TNF- α /IL-6R triple-specific fusion protein down-regulated in BUN expression more prominently.

3.5 Anti-TNF- α /IL-6R triple-specific fusion protein inhibits inflammatory mediators in the serum of septic rats

Cytokines, including IL-1 β , IL-6 and TNF- α , have been identified as key mediators in sepsis. In our experiment, these biomarkers were selected to demonstrate the therapeutic effects of anti-TNF- α /IL-6R triple-specific fusion protein against sepsis as shown in Fig. 3c-e. Satisfactorily, anti-TNF- α /IL-6R triple-specific fusion protein treatment significantly reduced these inflammatory mediators in a dose dependent manner. The single-targeted IL-6R or TNF- α fusion protein also had effect on down regulating these cytokines, although the effects were significantly weaker than anti-TNF- α /IL-6R triple-specific fusion protein at the same dose.

3.6 Anti-TNF- α /IL-6R triple-specific fusion protein alleviates histopathological lesions in the kidney of CLP rats

Acute kidney injury (AKI) is a serious complication of sepsis which occurs frequently, particularly in the elderly. The kidneys of different groups of rats were collected for histopathological analysis. In the sham operation group, the structure of the renal tubules and glomeruli was normal, with no damage. (Fig. 4a). The histopathological changes of the CLP group including marked interstitial hemorrhage and renal proximal tubular congestion, edema, necrosis and exfoliated cells are visible (Fig. 4b). In contrast, these lesions were obviously attenuated in the group with anti-TNF- α /IL-6R triple-specific fusion protein treatment in a dose-dependent manner (Fig. 4c-e) and minimal lesions were seen in anti-TNF- α /IL-6R triple-specific fusion protein (10 mg/kg) - treated CLP rat (Fig. 4e). The histologic features were less

severe and extensive in the rats treated by the single-targeted TNF- α (Fig. 4f) or IL-6R (Fig. 4g) fusion protein, through their relieving effects were significantly weaker than that of anti-TNF- α /IL-6R triple-specific fusion protein at the same dose. The bar graph showed that all the treatment groups had the therapeutic effect compared to CLP group.

Pro-inflammatory cytokine levels were also detected in kidney tissue. Compared with the sham operation group ($P < 0.01$), the levels of TNF- α , IL-1 β and IL-6 in the CLP group were significantly increased, while the fusion protein group was significantly decreased (Fig. 4h). The anti-TNF- α /IL-6R triple-specific fusion protein ($10 \text{ mg}\cdot\text{kg}^{-1}$ and $5 \text{ mg}\cdot\text{kg}^{-1}$) administration markedly decreased all of the three cytokines ($P < 0.01$, $P < 0.01$ vs CLP). As shown in Fig. 4i, the mRNA transcription level of IL-1 β , TNF- α and IL-6 in CLP group was significantly elevated, compared with the sham group. All fusion protein treated groups decreased the expression level of these inflammatory mediators, where the anti-TNF- α /IL-6R triple-specific fusion protein remarkably inhibited these gene expressions in a dose-dependent manner.

4 Discussion

Sepsis caused by the host response to infection is a life-threatening organ dysfunction that can significantly extend the length of hospitalization of patients [15, 16]. However, the underlying mechanism of sepsis is complicated and still unclear, thus the clinical treatment of sepsis is short of medicines. It is widely accepted that the extensive releasing of cytokines and other mediators like TNF- α and IL-6 are valuable biomarkers in septic condition, which can not only be employed to evaluate the prognosis of sepsis [6], but also be the therapeutic target to develop the medicines against sepsis.

In this study, we firstly constructed triple-specific fusion protein by linking 3 single domain chain, anti-TNF- α /HAS/IL-6R, which can block the functions of TNF- α and IL-6 under the septic experimental condition at the same time. Anti-serum albumin (HSA) moiety single-domain chain involved in this anti-TNF- α /IL-6R triple-specific fusion protein can extend the half-lives in vivo that its pharmacokinetic behavior can also be improved [14]. Based on the new prokaryotic expression system process developed in our lab, the triple-specific fusion protein allows for efficient expression of the excellent soluble and stable molecule in *E.coli* in large quantities with high purity over 95%, and without compromising its properties. The obtained triple-specific fusion protein displayed high binding affinity with TNF- α (94.75 nM), HSA (1.83 nM) and IL-6R (2.29 nM), analyzed by ELISA binding assay. In vitro, the anti-TNF- α /IL-6R triple-specific fusion protein can high-efficiency protect L929 cells from the apoptosis effects induced by TNF- α .

In our CLP septic model rats, the level of serum TNF- α , IL-1 β and IL-6, as promised biomarkers of sepsis, is significantly increased which indicated that sepsis had been initiated. After intervention treatment by the anti-TNF- α /IL-6R triple-specific fusion protein alone, the survival rate of CLP is significantly increased compared with the 10% survival rate of CLP groups within 72-hour. To compare with the single-targeted IL-6R or TNF- α fusion protein, the same dose of anti-TNF- α /IL-6R triple-specific fusion protein can increase the survival rate of CLP more significantly. Meanwhile, serum urea nitrogen (BUN), TNF- α , IL-1 β

and IL-6 collected from different CLP sepsis rat groups presented that triple-specific fusion protein can decrease the cytokine mediators more notably than monospecific fusion protein at the same concentration, supplying more evidences to support that anti-TNF- α /IL-6R triple-specific fusion protein have more potential treatment effects on sepsis.

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, where the important organs, specifically heart, lung and kidney, can be seriously damaged under septic condition [2]. In our experiment, the rat kidneys collected from the CLP group displayed histopathological changes including marked interstitial hemorrhage and renal proximal tubular congestion, edema, necrosis and exfoliated cells are visible. After the treatment by anti-TNF- α /IL-6R triple-specific fusion protein, the lesions appeared in untreated-CLP group were significantly attenuated by histopathological analysis. Levels of TNF- α , IL-1 β and IL-6 in detected in the kidney tissue by ELISA assays is meaningfully decreased in anti-TNF- α /IL-6R triple-specific fusion protein-treated CLP rat groups, and following the expression level of these inflammatory mediators were confirmed by RT-PCR in the gene level.

In conclusion, the triple-specific fusion protein presented potent therapeutic effects on septic animal model in this study, indicating that blocking the bioactivities of important inflammatory mediators, TNF- α and IL-6, at the same time can be potential assistant treatment method co-worked with antibiotic drugs in the sepsis clinical treatment. Continued studies in this direction in animal experiments and clinical trial under septic condition have the potential to shed light on the treatment for sepsis.

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Fujian Medical University (No. 2018-104). All animals were raised in the Laboratory Animal Center of Fujian Medical University (Certificate No. SCXK (Fujian) 2016-0006), where the animal work has taken place and animal handling procedures were performed in strict accordance with the care of laboratory animals, according to the Fujian Province Zoological Society.

Consent for publication

The manuscript is approved by all authors for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by National Natural Science Foundation, China (Grant No. 81402842), The outstanding Talents Training Project of the Educational Office of Fujian Province, China—Grant No. 2017052, Fujian provincial health technology project, China (Grant No. 2018-ZQN-61, 2018-ZQN-64) , Natural Science Foundation of Fujian Province, China (Grant No. 2019J01306, 2020J01634), The Emergency Project on New Coronavirus Pneumonia Prevention Research of Fujian Medical University (Grant No. 2020YJ004).

Author information

Xiaole Chen and Shuangyu Tan contributed equally to this work

Affiliations

Department of Bioengineering and Biopharmaceutics, School of Pharmacy, Fujian Medical University, Fuzhou City, 350122 Fujian Province, China

Xiaole Chen, Shuangyu Tan, Mengru Yan, Kaimei Nie, Qingmei Zheng, Yaduan Wang, Rui Liu & Juhua Yang

Department of Pharmacology, School of Pharmacy, Fujian Medical University, Fuzhou City, 350122 Fujian Province, China

Nanwen Zhang

School of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou City, 350122 Fujian Province, China

He Wang

Authors' contributions

Conceptualization, Juhua Yang and Xiaole Chen; methodology, Xiaole Chen and Nanwen Zhang; software, Rui Liu and Kaimei Nie; validation, Qingmei Zheng, Yaduan Wang and Mengru Yan; writing—original draft preparation, Xiaole Chen and Shuangyu Tang; writing—review and editing, He Wang and Nanwen Zhang; supervision, He Wang and Juhua Yang; project administration, Kaimei Nie; funding acquisition, He Wang, Xiaole Chen and Nanwen Zhang.

Corresponding author

Correspondence to Nanwen Zhang, He Wang or Juhua Yang.

Acknowledgements

The authors wish to thank Fujian Medical University for providing their equipment to conduct the experiment.

References

- [1] Skirecki T, Cavaillon J. M. 2019. Inner sensors of endotoxin – implications for sepsis research and therapy. *FEMS Microbiology Reviews* 43:239-256
- [2] Finfer S., Machado, F.R. 2016. The Global Epidemiology of Sepsis. Does It Matter That We Know So Little?. *American journal of respiratory and critical care medicine* 193: 228-230.
- [3] Singer M., Deutschman C.S., Seymour C.W., Shankar-Hari M., Annane D., Bauer M., Bellomo R., Bernard G.R., Chiche J.D., Coopersmith C.M., Hotchkiss R.S., Levy M.M., Marshall J.C., Martin G.S., Opal S.M., Rubenfeld G.D., van der Poll T., Vincent J.L. Angus D.C. 2016. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *Jama* 315: 801-810.
- [4] Hotchkiss R.S., Karl I.E. 2003. The pathophysiology and treatment of sepsis. *The New England journal of medicine* 348: 138-150.
- [5] Pantović-Stefanović M., Petronijević N., Dunjić-Kostić B., Velimirović M., Nikolić T., Jurišić V., Lačković M., Damjanović A., Totić-Poznanović S., Jovanović A.A., Ivković M. 2018. sVCAM-1, sICAM-1, TNF- α and IL-6 levels in bipolar disorder type I: Acute, longitudinal and therapeutic implications. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry* 19: S41-S51.
- [6] Song R., Kim J., Yu D., Park C., Park J. 2012. Kinetics of IL-6 and TNF- α changes in a canine model of sepsis induced by endotoxin. *Veterinary immunology and immunopathology* 146: 143-149.
- [7] Alicja K. P., Aleksandra C.T., Agnieszka S.K., Maciej K. B., Alina B. 2020. Biochemical Parameters in Cognitive Functions. *Neuropsychiatric Disease and Treatment*. 16:2479-2489.
- [8] Kang S., Tanaka T., Kishimoto T. 2015. Therapeutic uses of anti-interleukin-6 receptor antibody. *International immunology* 27: 21-29.
- [9] Rau S., Kohn B., Richter C., Fenske N., Küchenhoff H., Hartmann K., Härtle S., Kaspers B., Hirschberger J. 2007. Plasma interleukin-6 response is predictive for severity and mortality in canine systemic inflammatory response syndrome and sepsis. *Veterinary clinical pathology* 36: 253-260.
- [10] Watanabe E., Hirasawa H., Oda S., Matsuda K., Hatano M., Tokuhisa T. 2005. Extremely high interleukin-6 blood levels and outcome in the critically ill are associated with tumor necrosis factor- and interleukin-1-related gene polymorphisms. *Critical care medicine* 33 : 89-97; discussion 242-243.
- [11] Hamers-Casterman C., Atarhouch T., Muyldermans S., Robinson G., Hamers C., Songa E.B., Bendahman N., Hamers R. 1993. Naturally occurring antibodies devoid of light chains. *Nature* 363: 446-

[12] Arbabi-Ghahroudi M. 2017. Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook. *Frontiers in immunology* 8: 1589.

[13] Muyldermans S. 2013. Nanobodies: natural single-domain antibodies. *Annual review of biochemistry* 82: 775-797.

[14] Kontermann R.E. 2012. Dual targeting strategies with bispecific antibodies. *mAbs* 4: 182-197.

[15] Fleischmann C., Scherag A., Adhikari N.K., Hartog C.S., Tsaganos T., Schlattmann P., Angus D.C., Reinhart K. 2016. Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations. *American journal of respiratory and critical care medicine* 193 : 259-272.

[16] Hotchkiss R.S., Monneret G., Payen D. 2013. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *The Lancet. Infectious diseases* 13: 260-268.

Figures

Figure 1

Figure 1

The preparation of anti-TNF-α/IL-6R triple-specific fusion protein. (a) 3D structure of an anti-TNF-α/IL-6R triple-specific fusion protein mimicked by SWISS-MODEL. (b) The colony PCR of anti-TNF-α/IL-6R (c) The

expression product of anti-TNF- α /IL-6R triple-specific fusion protein. Lane M, marker; lane 1: BL21(DE3); lane 2: BL21(DE3)/ anti-TNF- α /IL-6R triple-specific fusion protein, lane 3: supernatant; lane 4: sediment. (d) The process of purification. Lane M, marker; lane 1: BL21(DE3); lane 2: BL21(DE3)/ anti-TNF- α /IL-6R triple-specific fusion protein, lane 3: supernatant; lane 4: sediment, Lane 5-6: washed IBs, Lane 7: renaturation protein, Lane 8: Further purified protein by one-step BeaverBeads™ IDA-Nickel, Lane 9: Desalting. (e) Desalting with Äkta. (f) Binding capacity analysis of anti-TNF- α /IL-6R triple-specific fusion protein.

Figure 2

Figure 2

Inhibition of TNF- α cytotoxicity using anti-TNF- α /IL-6R triple-specific fusion protein. (A) MTT assay for the abilities of the anti-TNF- α /IL-6R triple-specific fusion protein to inhibit TNF- α activity. (B) The cells were cultured with 2ng/ml TNF α and 10ug/ml ActionmycinD in the presence of anti-TNF- α /IL-6R fusion protein (32Nm, 3.2nM, 0.32nM, 0.032nM) and then the cells were processed for DAPI/PI double stain assay. DAPI was used to stain the nuclei. PI was used to stain apoptotic cells. Images were taken under fluorescent microscope (DAPI/PI at $\times 10$). a.control, b.TNF α , c.TNF α +32nM anti-TNF- α /IL-6R fusion protein, d.TNF α +3.2nM anti-TNF- α /IL-6R fusion protein, e. TNF α +0.32nM anti-TNF- α /IL-6R fusion protein, f. TNF α +0.032nM anti-TNF- α /IL-6R fusion protein. (C) Apoptosis rate analysis. All data are represented as means $\pm$ SD, *P < 0.05, **P < 0.01, ***P < 0.001 vs TNF- α group, ###P < 0.001 vs the control group.

Figure 3

Figure 3

Anti-TNF- α /IL-6R triple-specific fusion protein significantly alleviates CLP induced sepsis. (a) Survival proportions of septic rats. (b) The expression levels of septic rats. Reduction of key inflammatory-related mediators in the serum of septic rats after the treatment. The serum was collected after the sacrifice of rats and analyzed by commercial ELISA kits. The concentration was represented by bar graphs. (c) IL-6 (d) TNF- α (e) IL-1 β . All data are represented as means SD, *P < 0.05, **P < 0.01, ***P < 0.001 vs CLP group, ###P < 0.001 vs sham group.

Figure 4

Figure 4

Anti-TNF- α /IL-6R fusion protein alleviates histopathological lesions in septic rats. (a) Sham; (b) CLP; (c) CLP + anti-TNF- α /IL-6R 1mg·kg⁻¹; (d) CLP + anti-TNF- α /IL-6R 5mg·kg⁻¹; (e) CLP + anti-TNF- α /IL-6R 10mg·kg⁻¹; (f) CLP + anti-TNF- α 5mg·kg⁻¹; (g) CLP + anti-IL-6R 5mg·kg⁻¹. Anti-TNF- α /IL-6R triple-specific fusion protein down-regulates the expression of inflammatory cytokines in the kidney of CLP group. (h) IL-6, TNF- α and IL-1 β expression, **P < 0.01, ***P < 0.01 vs CLP group, ###P < 0.001 vs sham group. (i) Anti-TNF- α /IL-6R triple-specific fusion protein inhibits the mRNA expression of inflammatory mediators in the kidney, relative mRNA of IL-6, relative mRNA of TNF- α and relative mRNA of IL-1 β , *P < 0.05, **P < 0.01, ***P < 0.01 vs CLP, ###P < 0.001 vs sham group. Scale bars, 50 μ m(a-g).