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ORIGINAL RESEARCH

Zhike Erfang Alleviates MRSA-Induced Pneumonia by Inhibiting TRAF6 and Activating NLRP3 Inflammatory Body

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Purpose: The therapeutic effects of Zhike Erfang in modulating the cellular responses and immune microenvironment associated with MRSA-induced acute lung injury remain unclear. This study aims to elucidate the potential mechanisms by which Zhike Erfang mitigate the cellular and molecular effects of MRSA in a laboratory model.

Patients and Methods: A mouse model of acute lung injury was established using heat-inactivated MRSA. Lung tissue and bronchoalveolar lavage fluid were collected for analysis. Macrophages were pretreated with Zhike Erfang for 30 minutes before exposure to heat-inactivated MRSA for 24 hours. Protein expressions of TRAF6, iNOS, TNF-α, IL-1β, NLRP3, and caspase-1 in lung tissues were quantified using Western blot. The content of LDH was detected by the lactate dehydrogenase cytotoxicity test kit.

Results: Zhike Erfang significantly reduced the expression of iNOS, LDH, TNF-α, IL-1β, NLRP3, and caspase-1 in a dose-dependent manner in lung tissues from the MRSA model. Zhike Erfang inhibited the expression of TRAF6.

Conclusion: Zhike Erfang can alleviate pneumonia caused by MRSA by inhibiting TRAF6 and inducing NLRP3 inflammatory body activation.

Keywords: Zhike Erfang, methicillin-resistant Staphylococcus aureus, TRAF6, NLRP3, pneumonia

Introduction

Pneumonia is a global disease caused by infectious bacteria.[1] Multi-drug-resistant bacteria are significant contributors to the morbidity and mortality of pneumonia patients, among which MRSA plays a corresponding role.[2] Methicillin-resistant Staphylococcus aureus (MRSA) is considered one of the most common and dangerous antibiotic-resistant bacteria in hospital-acquired infection, causing diseases such as pneumonia, infectious arthritis, osteoarthritis and bacteremia.[3] Over the years, the increasing use of antibiotic therapy for MRSA has led to the development of various drug-resistant survival mechanisms, including cell wall thickening, increased efflux pumps, drug target mutation, enzymatic drug modification and biofilm formation. These mechanisms have contributed to numerous adverse clinical outcomes[4] The pneumonia caused by MRSA lacks effective means of treatment, underscoring the urgent need for novel therapeutic strategies.[5,6] Therefore, it is of great significance to explore anti-inflammatory drugs for MRSA-induced inflammatory reactions and reveal their effective targets for treating pneumonia caused by MRSA.

Traditional Chinese medicine (TCM) offers a unique perspective on the pathogenesis of lung diseases, attributing conditions such as pneumonia to an imbalance of the lung, spleen, and kidney qi, with phlegm obstructing the lungs. This pathophysiological framework supports the TCM principle of "treating different diseases with the same treatment", particularly through the use of compound Chinese medicines. In this context, the formula "Zhike Er Fang" was

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developed by Professor Huang Jigeng of Shuguang Hospital, who drew upon decades of clinical experience and insights from the classical TCM text "Yixue Xinwu.[7,8] The formula is composed of Cicada Slough (Chan Yi), Stiff Silkworm (Jiang Can), Almond (Xing Ren), Peucedanum (Qian Hu), Belamcanda (She Gan), Bupleurum (Chai Hu), Scutellaria (Huang Qin), hogfennel root, Willowleaf Swallowwort Rhizome, Aster, Pinellia Ternata, Blackberrylily Rhizome, Chinese Thorowax Root, Aurantii Fructus, Platycodon Grandiflorum, liquorice root, Tatarian Aster Root, and Farfara honey.[9] It is designed to address the fundamental disease mechanism in pneumonia—" the mutual obstruction of wind-evil and phlegm-heat in the lungs, and the loss of lung qi dispersal and descent, with a therapeutic strategy of "expelling wind, clearing heat, ventilating the lungs, and stopping cough". Our formula has shown promising results in preliminary clinical trials, including those conducted by Dr. Wu Kunlun, head of the TCM department at our hospital.[10] However, the pharmacological mechanisms underlying its anti-inflammatory actions remain under-investigated. This study aims to elucidate these mechanisms, providing a scientific basis for the rational use of Zhike Er Fang in the intervention of pneumonia. The botanical components of Zhike Er Fang were formally identified by the botanist Dr. Liu Mingyu, ensuring the authenticity and consistency of our herbal preparations.[11] This rigorous botanical verification further strengthens the scientific foundation of our study, aligning with the high standards required for the effective exploration of TCM in treating complex diseases like MRSA-induced pneumonia.

The NLRP3 inflammasome is crucial in the innate immune response, sensing pathogens and stress signals. Comprising the NLRP3 sensor, ASC adaptor, and pro-caspase-1, it activates pro-inflammatory cytokines IL-1β and IL-18.[12] MRSA infections prompt NLRP3 inflammasome activation chiefly through toxins like Panton-Valentine leukocidin (PVL) and α-hemolysin. The presence of α-hemolysin in Staphylococcus aureus activates NLRP3 inflammatory corpuscles during pneumonia infection and induces necrotizing lung injury.[13] Importantly, NLRP3 (-/-) mice suffer from less severe symptoms of pneumonia.[14] Furthermore, PVL, a pore-forming toxin in Staphylococcus aureus, may also lead to life-threatening necrotizing infection. PVL binds monocytes and macrophages to activate NLRP3 inflammatory corpuscles and participate in the release of caspase-1-dependent pro-inflammatory cytokines IL-1β and IL-18.[12] Moreover, according to literature reports, α-hemolysin and pore-forming toxin in MRSA may activate NF-κB and then affect the activation of NLRP3, thus regulating tissue damage.[15] Additionally, variations in NLRP3 expression, influenced by factors such as nutritional status, highlight the complexity of its regulatory mechanisms in MRSA infections.[16] TNFAIP3 (A20) is a TRAF6 enzyme that can regulate the production of anti-inflammatory mediators and alleviate LPS-induced lung inflammation.[17] A previous study reported that TLRs stimulate the formation of the NLRP3 inflammasome, which plays a central role in the body's defense against Staphylococcus aureus.[18] Given the intricate role of the NLRP3 inflammasome in MRSA-induced inflammation and the limited therapeutic options that directly modulate this pathway, our research investigates Zhike Erfang's potential as a novel intervention, bridging a significant gap in current treatment strategies.

Based on the above research, we speculate that Zhike Erfang may alleviate the inflammatory reaction caused by MRSA in vivo and in vitro through TRAF6 and NLRP3 inflammasome activation. Therefore, this study systematically clarified the effect of Zhike Erfang on MRSA-induced inflammation from experimental animals, cell function and molecular mechanism, and explores whether its mechanism is associated with TRAF6 and NLRP3 inflammatory corpuscle activation.

Materials and Methods

Source and Composition of Chinese Medicine Compound

The Zhike Erfang was summarized by Professor Huang Jigeng of Shuguang Hospital, who has many years of clinical experience, taking the meaning of Zhissou powder and Xiaochaihu decoction in "Medical Heart wu". It consists of Cicada 9 g, Stiff Silkworm 9 g, Apricot Seed 9 g, hogfennel root 12 g, Willowleaf Swallowwort Rhizome 12 g, Aster 12 g, Pinellia Ternata 12 g, Blackberrylily Rhizome 12 g, Chinese Thorowax Root 15 g, Scutellaria 15 g, Aurantii Fructus 12 g, Platycodon Grandiflorum 9 g, liquorice root 9 g, Tatarian Aster Root 15 g, Farfara honey 15 g. Aiming at the basic pathogenesis of "the mutual resistance of wind and phlegm and heat, and the loss of lung qi in Xuansu". It is formulated with the treatment principle of "dispelling wind and clearing away heat, Xuanfei and relieving cough".

Bacterial Culture and Growth

In our study, the acute lung injury model in mice was induced using a specific strain of methicillin-resistant *Staphylococcus aureus* (MRSA), USA300. Briefly, this strain was grown to mid-log phase (OD 600 nm) in Luria-Bertani (LB) with shaking at a rate of 200 rpm in a constant temperature incubator at 37 °C, ensuring consistent growth conditions. Prior to use in experiments, the bacterial cultures were heat-inactivated by heating at 60°C for 1 hour, a method confirmed to effectively eliminate bacterial viability while preserving antigenicity for immune response studies.

Animal

The acute lung injury model in mice was induced by methicillin-resistant Staphylococcus aureus (MRSA). Male C57/BL6 mice, aged 6–8 weeks, were acquired from Beijing Huafukang Biotechnology Co., LTD (License number: SCXK (Beijing) 2020–0004), and acclimatized in a specific sterile environment (25°C, humidity 55%). The procedures in this study were conducted under the stringent guidelines approved by the Institutional Biosafety Committee (IBC) at Gongli Hospital in Pudong New District, Shanghai. The medical ethics statement of relevant animal experiments was approved by the Shanghai Municipal Health Commission (SN.No2023121, approval date 15 January 2020).

The administration of Zhike Erfang by gavage commenced three days prior to MRSA challenge to establish sufficient bioactive levels, with daily doses at low, medium, and high concentrations (12, 24, 48 g/kg/d). Each mice were anaesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, and standardized suspension containing 1×10^8 colony-forming units (CFU) of heat-inactivated MRSA was given to mice by tracheal instillation with an indwelling needle. The established mouse model of MRSA pneumonia was monitored continuously for the first 4 hours, then checked once every six hours for clinical signs of severity, including ruffled fur, markedly decreased activity, surface temperature < 23°C, diminished righting reflex and decreased respiratory rate. Manifestations, indicative of profound illness, were most pronounced at the 24-hour mark, compelling the decision to euthanize the animals at this juncture with 150 mg/kg pentobarbital, and Alveolar lavage fluid and lung tissue were collected.

Cell Culture

Isolation, Culture, and Stimulation of Macrophages: Alveolar macrophages were isolated via three bronchoalveolar lavages using DPBS. Simultaneously, bone marrow-derived macrophages (BMDMs) were harvested from the femurs and tibias of mice. For both types, cells were collected by centrifugation at 300 x g for 10 minutes, followed by a 3-minute red blood cell lysis using ammonium chloride. After a second centrifugation under the same conditions, the alveolar macrophages were seeded in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and allowed to adhere for 1 hour, while bone marrow cells were cultured in RPMI 1640 medium with 20% FBS and 20 ng/mL M-CSF for 7 days to differentiate into macrophages.

After 30 minutes of adherence, the macrophages were pre-treated with Zhike Erfang, prepared at a concentration of 10 μg/mL in the medium 24 hours before stimulation. Following this, the Alveolar macrophages were stimulated by heat-inactivated MRSA (MOI=200) for 24 h, while the BMDM was stimulated with the NLRP3 inflammasome inducers nigericin at a concentration of 10 μM for 1 hour. The expression of iNOS was detected by Western Blot. The expressions of TNF-α and IL-1β at the protein level were detected by Western blotting. The level of LDH produced by cells was detected by the lactate dehydrogenase cytotoxicity test kit (C0016, Beyotime Biotechnology, China).

HE Staining

HE staining kit (C0105S, Beyotime Biotechnology, China) was used for pathological examination. The lung tissue were collected from the model mice and fixed in 4% formaldehyde for 24 h. After gradient alcohol dehydration, tissues were cleared in xylene and soaked in the wax solution for 24 h. The embedded tissues were sectioned into slices approximately 5 μm thick. Following xylene dewaxing and gradient alcohol rehydration, hematoxylin-eosin dyeing was carried out. After dehydration with gradient alcohol and clearing in xylene, the sections were mounted with neutral gum. The dyeing results were observed under the microscope (CK53, Olympus, Japan) after air drying.

Western Blotting

Proteins were extracted from lung lavage fluid, tissues, and cell cultures, and their concentrations were determined after treatment with lysis buffer. The protein samples were then denatured by boiling at 100°C. Following denaturation, proteins were separated using electrophoresis on a 10% SDS-polyacrylamide gel and subsequently transferred onto a PVDF membrane for analysis. The membrane loaded with protein was incubated overnight at room temperature with primary antibodies, such as anti-iNOS (ab178945), anti-TNF-α (ab183218), anti-IL-1β (ab254360), anti-TRAF6 (ab40675), anti-NLRP3 (ab263899), caspase-1 (ab138483), and incubated for 4 h at room temperature with secondary antibody (ab288151, Abcam, USA) combined with primary antibody. After the PVDF membrane was treated with ECL luminous liquid, the membrane was exposed by the scanner in the darkroom. Finally, a semi-quantitative analysis of the bands was carried out with Image J software.

BCA

The lung tissue was homogenized in 0.5% cetyltrimethyl ammonium bromide (HTAB), and the supernatant was resuspended in HTAB. After twice freezing and thawing, homogenization and centrifugation, the supernatant was collected, and the protein concentration was determined by BCA method. TMB and H_2O_2 were added to the supernatant, and the change of absorbance per minute at the wavelength of 655 nm was read by the enzyme-labelled instrument as a unit of the enzyme. The activity of myeloperoxidase in tissue was expressed by the enzyme content per gram of protein.

Quantitative RT-PCR

A total RNA from cells and tissue samples was extracted using TRIzol (Cat# B511311, Sangon Biotech). After the reverse transcription, the quantitative real-time PCR analyses were performed with 10 μL reactions using SYBR Premix Ex Taq II (TaKaRa Biotechnology, Dalian, China) on a LightCycler 480 system (Roche, Basel, Switzerland). The thermal cycling conditions included an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. β-actin was employed as an internal control to normalize the expression data. Relative gene expression levels were calculated using the 2−ΔΔCT method. Primer sequences used for the amplification of target genes are listed in Table 1.

Table 1 List of the PCR Primer Sequences Used in Our Study

Name	Sequence
NLRP3	Forward: ATGCTGCTTCGACATCTCCT
	Reverse: AACCAATGCGAGATCCTGAC
Caspase-1	Forward: CACAGCTCTGGAGATGGTGA
	Reverse: TCTTTCAAGCTTGGGCACTT
IL-1β	Forward: GCCCATCCTCTGTGACTCAT
	Reverse: AGGCCACAGGTATTTTGTCG
GAPDH	Forward: GACATCAAGAAGGTGGTGAAGC
	Reverse: GAAGGTGGAAGAGTGGGAGTT
TRAF6	Forward: CATCTTCAGTTACCGACAGCTCAG
	Reverse: TGGTCGAGAATTGTAAGGCGTAT
TNF-α	Forward: CTCACACTCAGATCATCTTCTC
	Reverse: CTTTCTCCTGGTATGAGATAGC

Statistical Analysis

GraphPad Prism 9 was used for data analysis and graphical production, with measured value expressed as Mean ±Standard Deviation. Analysis of variance was used for comparison among groups, and P<0.05 showed that the difference was statistically significant.

Results

Zhike Erfang Relieves Pulmonary Inflammation Caused by MRSA

Zhike Erfang significantly mitigates pulmonary inflammation induced by MRSA, as evidenced by histological assessments using hematoxylin and eosin (HE) staining. In the MRSA group, extensive pathological alterations were evident, including focal bronchitis, diffuse interstitial pneumonia, pronounced infiltration of inflammatory cells, and vascular dilation and congestion. These changes culminated in substantial destruction of the alveolar structures, indicative of severe inflammatory responses. Conversely, treatment with Zhike Erfang resulted in a marked decrease in the infiltration of inflammatory cells within the alveolar spaces. Moreover, the integrity of the bronchial walls was largely preserved. Notably, the extent of these improvements was dose-dependent, with higher concentrations of Zhike Erfang yielding more pronounced alleviation of the MRSA-induced pulmonary damage (Figure 1A). The lung injury scores, as quantified and graphed in Figure 1B, underscore a dose-dependent improvement in lung tissue condition. This concentration-dependent effect underscores the potential of Zhike Erfang in restoring lung tissue integrity and function following MRSA-induced injury.

Zhike Erfang Relieves Pulmonary Inflammation Caused by MRSA

Inducible nitric oxide synthase (iNOS) is a key enzyme in inflammation and oxidative stress.[19] Therefore, Western blot was used to detect the expression of iNOS in lung tissue. The results showed that the iNOS expression was significantly reduced in the lung tissues treated with Zhike Erfang compared to the MRSA control group. This reduction was notably dose-dependent, with higher concentrations of Zhike Erfang showing greater decreases in iNOS expression (Figure 2A and B). This suggests that Zhike Erfang effectively mitigates nitrosative stress within the lung tissue in a concentration-sensitive manner. Next, lung tissue and lung lavage fluid were taken to detect the expression of inflammatory factors TNF-α. Similar to iNOS, TNF-α levels in lung tissues were markedly lowered in the Zhike Erfang-treated groups, with a pronounced suppression observed in higher treatment concentrations (Figure 2A and C), highlighting Zhike Erfang's potent anti-inflammatory effects on cytokine modulation in lung tissues. Myeloperoxidase (MPO) mainly exists in the eosinophil granules of neutrophils and is expressed on the surface of neutrophils.[20] Activation of neutrophil

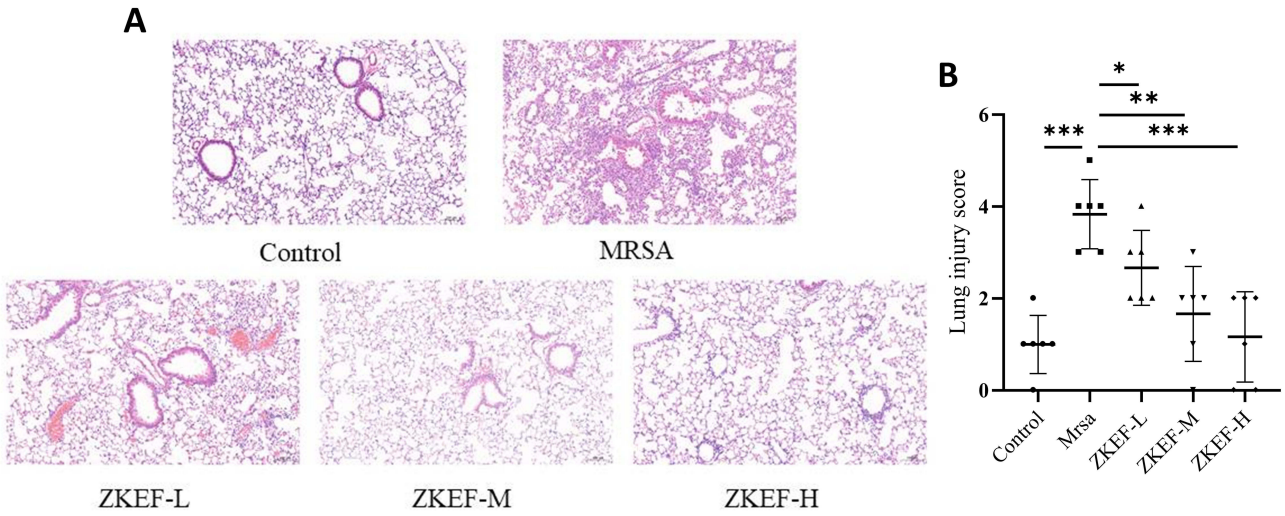

Figure 1 Zhike Erfang improve the pathological damage of lung tissue induced by MRSA. (**A**) HE staining was used to observe the improvement effect of Zhike Erfang on pneumonia caused by MRSA. (**B**) The graphical quantification of the Lung tissue injury in mouse model. *P<0.05, **P<0.01, ***P<0.001.

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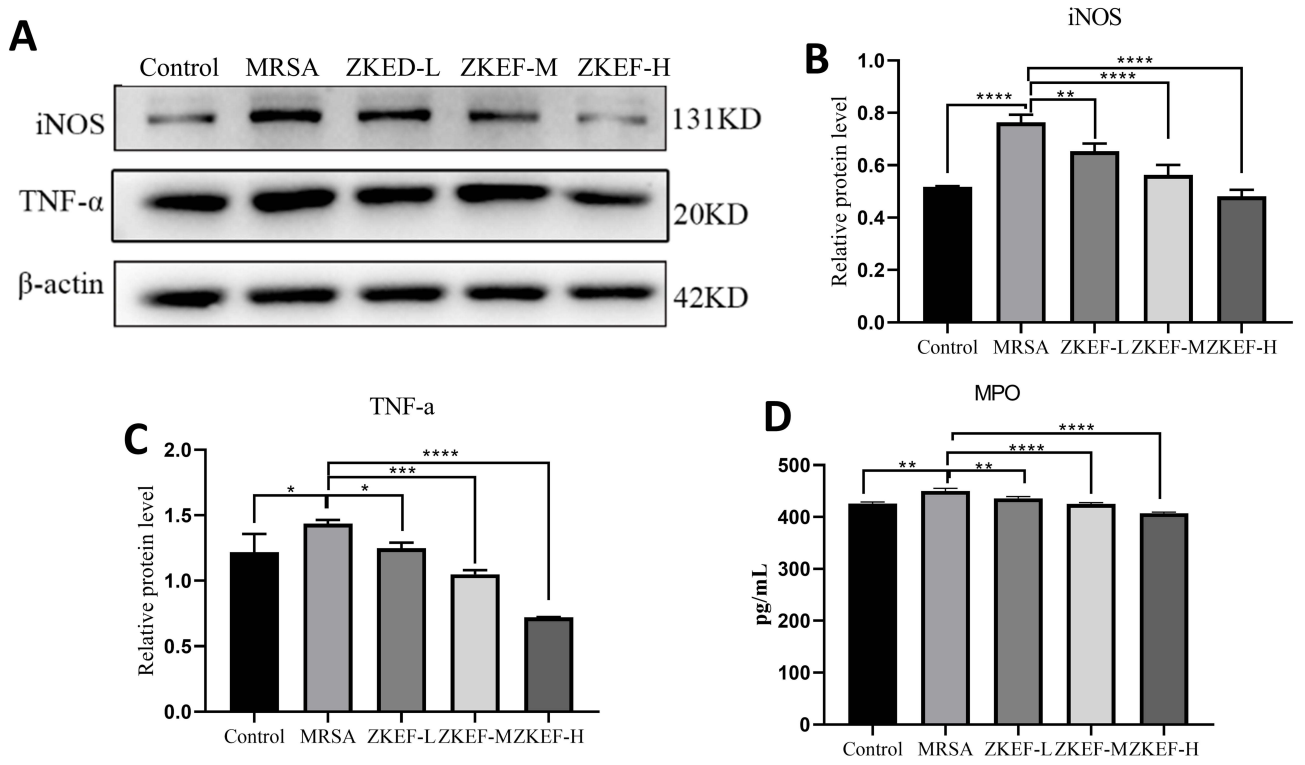

Figure 2 Zhike Erfang relieves pulmonary inflammation caused by MRSA. (**A**) Western blot was used to detect the expression of iNOS and TNF-α in lung tissue. (**B**) Quantification of protein expression of iNOS. (**C**) Quantification of protein expression of TNF-α. (**D**) Myeloperoxidase (MPO) content was detected by (bicinchoninic acid) BCA method. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

degranulation releases MPO, which has strong peroxidase activity and participates in inflammatory damage of tissues and organs.[21] MPO was detected by the BCA method, and the expression of MPO in the lung lavage fluid of model mice was significantly lower than that in the model group in a concentration-dependent manner with Zhike Erfang (Figure 2D). These results collectively demonstrate the efficacy of Zhike Erfang in modulating key inflammatory and oxidative markers across varying concentrations, underscoring its potential as a therapeutic option for MRSA-induced pneumonia.

Zhike Erfang Regulates the Activation of Macrophages Stimulated by MRSA

Cell experiments were carried out to explore the internal effect of Zhike Erfang on MRSA injury. Our Western blot and qPCR data revealed that the MRSA-infected control group exhibited significantly elevated levels of IL-1β, which was rescued by Zhike Erfang, suggesting that Zhike Erfang effectively suppresses inflammasome activity, which is critical in managing lung inflammation induced by MRSA (Figure 3A, D and F). Similarly, Inducible nitric oxide synthase (iNOS) and Tumor necrosis factor-alpha (TNF-α) showed increased levels in the MRSA group. Treatment with Zhike Erfang reduced the expression of iNOS (Figure 3A and B) and TNF-α (Figure 3A, C and E), aligning with the observed decreases in IL-1β further confirming the anti-oxidative and anti-inflammatory properties of Zhike Erfang. This detailed description highlights the significant inhibitory effects of Zhike Erfang on several biochemical markers of inflammation in a model of MRSA-induced lung injury, providing a clear understanding of its potential mechanism of action in reducing pulmonary inflammation.

Zhike Erfang Inhibits TRAF6 and Induces NLRP3 Inflammatory Corpuscles to Relieve Lung Inflammation in a Concentration-Dependent Manner

TRAF6 is a ubiquitin ligase that acts as a tumor suppressor by ubiquitinating target genes and inhibiting their transcriptional activity.[22] Therefore, this study detected the expression of TRAF6 in lung tissue of mice with model

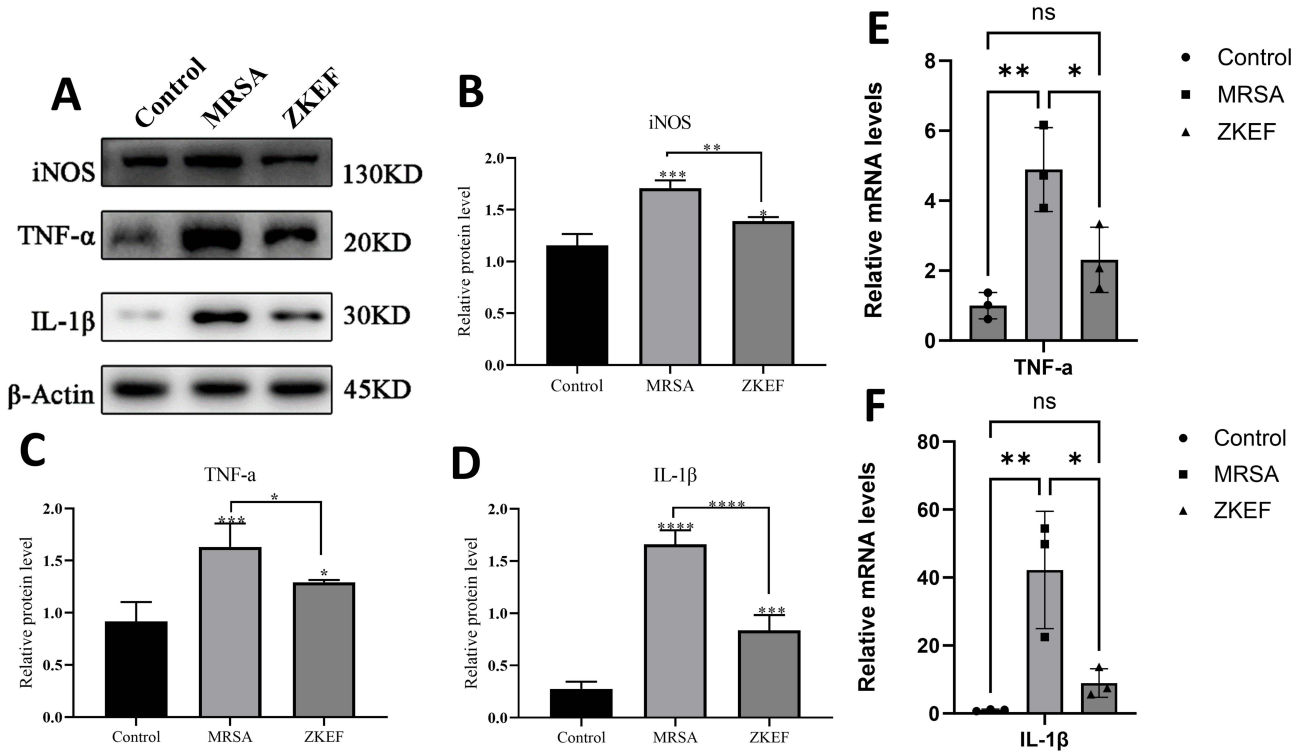

Figure 3 Zhike Erfang regulates the activation of macrophages stimulated by MRSA. (**A**) MRSA induced macrophages were taken to detect the expression of inflammatory factors TNF-α, iNOS and IL-1β by Western blot. (**B-D**) Quantification of protein expression of iNOS, TNF-α, and IL-1β in MRSA induced macrophages, respectively. (**E** and **F**) The qPCR determination of TNF-α, and IL-1β in MRSA induced macrophages. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

injury, and the findings revealed that Zhike Erfang inhibited the expression of TRAF6 (Figure 4A, D and G), suggesting that Zhike Erfang might alleviate lung inflammation by inhibiting TRAF6 expression.

NLRP3 inflammatory corpuscle is an important pattern recognition receptor for alveolar macrophages to exert anti-infection immunity, which induces the release of interleukin (IL)-1β and mediates the downstream inflammatory response.[23] A large number of studies show that NLRP3 inflammatory corpuscles play a protective role in anti-H1N1 virus immunity.[24–26] It has also been found that MRSA can activate NLRP3 inflammatory corpuscles through phagocytosis lysosomes. Therefore, the activation of NLRP3 inflammatory corpuscles induced by Zhike Erfang in injured lung tissue was detected with WB and RT-qPCR. The results showed that Zhike Erfang inhibited the expression of NLRP3 in model tissue compared with the model group in a concentration-dependent manner (Figure 4A, B and G). Moreover, the expression of associated inflammatory markers NF-κB p65, IL-1β, and caspase-1 were also reduced, as evident from the WB and qPCR analysis (Figure 4A, C and E–G). In conclusion, these findings confirm Zhike Erfang's robust anti-inflammatory effects in MRSA-induced lung inflammation, demonstrating its efficacy in inhibiting key proteins like TRAF6 and reducing NLRP3 inflammasome activity. This highlights its potential as a therapeutic strategy for severe pulmonary inflammatory conditions.

Specificity of Zhike Erfang in Inhibiting NLRP3 Inflammasome Activation in Bone Marrow-Derived Macrophages

We conducted targeted in vitro experiments to determine the specificity of Zhike Erfang's inhibitory effects on the NLRP3 inflammasome in bone marrow-derived macrophages (BMDMs) exposed to nigericin, a canonical NLRP3 activator. Exposure to nigericin significantly upregulated NLRP3, caspase-1, and IL-1β, confirming inflammasome activation in bone marrow-derived macrophages. Pre-treatment with Zhike Erfang markedly reduced these proteins, demonstrating an inhibitory effect (Figure 5B). Additionally, Zhike Erfang decreased NF-κB p65 expression, further indicating its broad anti-inflammatory properties (Figure 5A). These findings confirm that Zhike Erfang effectively

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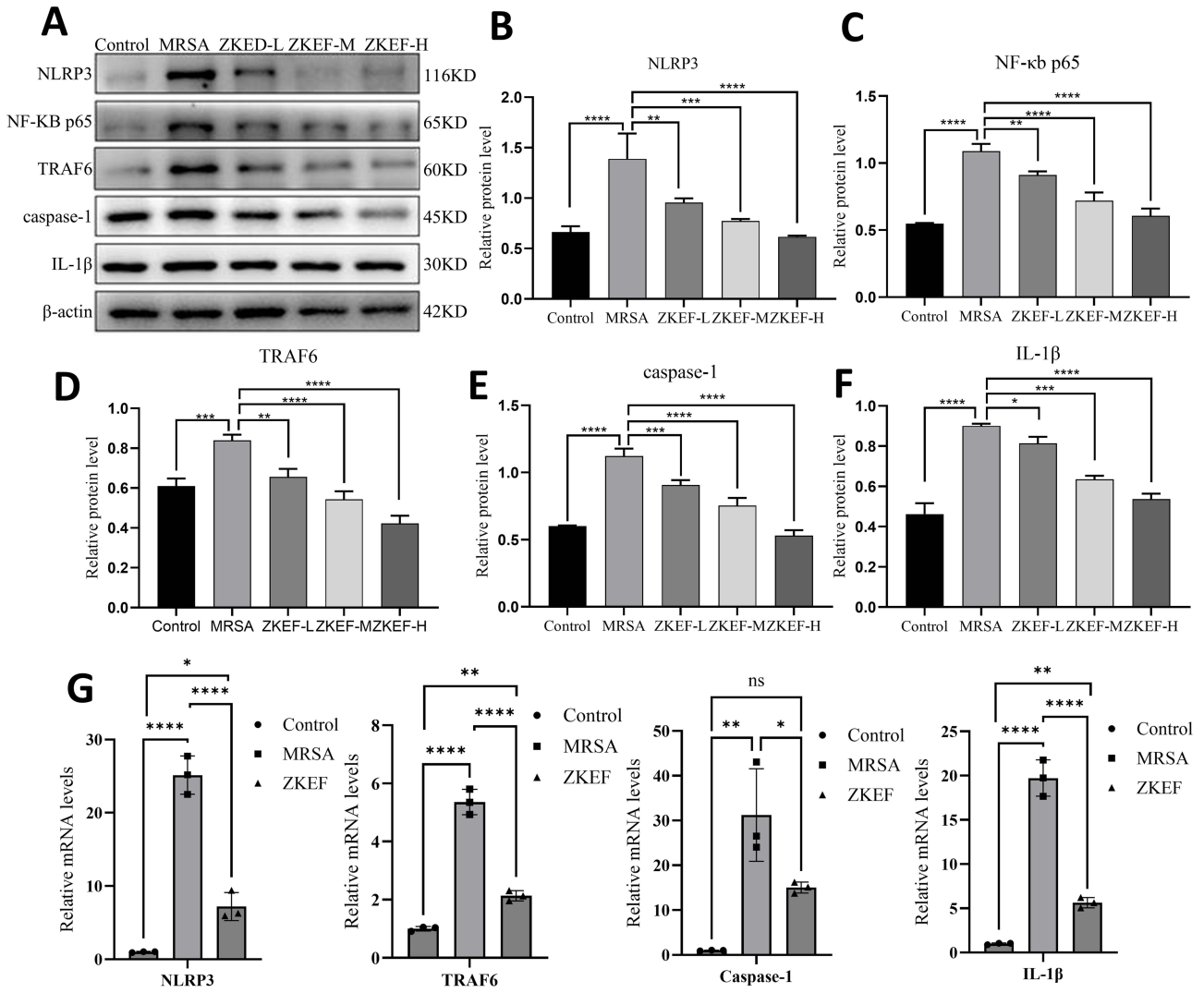

Figure 4 Zhike Erfang inhibits TRAF6 and induces NLRP3 inflammatory corpuscles to relieve lung inflammation in a concentration-dependent manner. (**A**) The expression of TRAF6, NLRP3, NF-κB p65, IL-1β and caspase-1 were measured by Western blot. (**B-F**) The protein expression of TRAF6, NLRP3, NF-κB p65, IL-1β, and caspase-1 was quantified by Western blotting. (**G**) qPCR analysis of TRAF6, NLRP3, IL-1β, and caspase-1. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

suppresses NLRP3 inflammasome activation and is not restricted to specific stimuli, suggesting its potential as a versatile anti-inflammatory agent. Future investigations will assess its efficacy against other inflammasome inducers to define its full therapeutic range.

Discussion

At present, the use of antimicrobials caused by pathogenic bacteria often leads to drug resistance, resulting in various adverse events. Now, with the continuous development, traditional Chinese medicine compounds have demonstrated significant therapeutic potential in pneumonia caused by pathogenic bacteria. For example, Lianhua Qingwen Capsule, which is composed of Maxing Shigan Decoction and Yinqiao Powder, can improve the immune regulation of Secondary Bacterial Europe.[27] Similarly, Buzhong Yiqi decoction, composed of Radix Astragali, Radix Ginseng, Radix Glycyrrhizae, Rhizoma Atractylodis Macrocephalae, Radix Angelicae Sinensis, etc., has shown high safety in treating pneumonia caused by drug-resistant bacteria.[28] However, there is no effective treatment for pneumonia caused by MRSA. Therefore, this study investigates Zhike Erfang,composed of almond, Peucedanum, Rhizoma Cynanchi Stauntonii, Rhizoma Pinelliae, Rhizoma Belamcandae, Radix Bupleuri, Scutellariae Radix, Fructus Aurantii, Radix

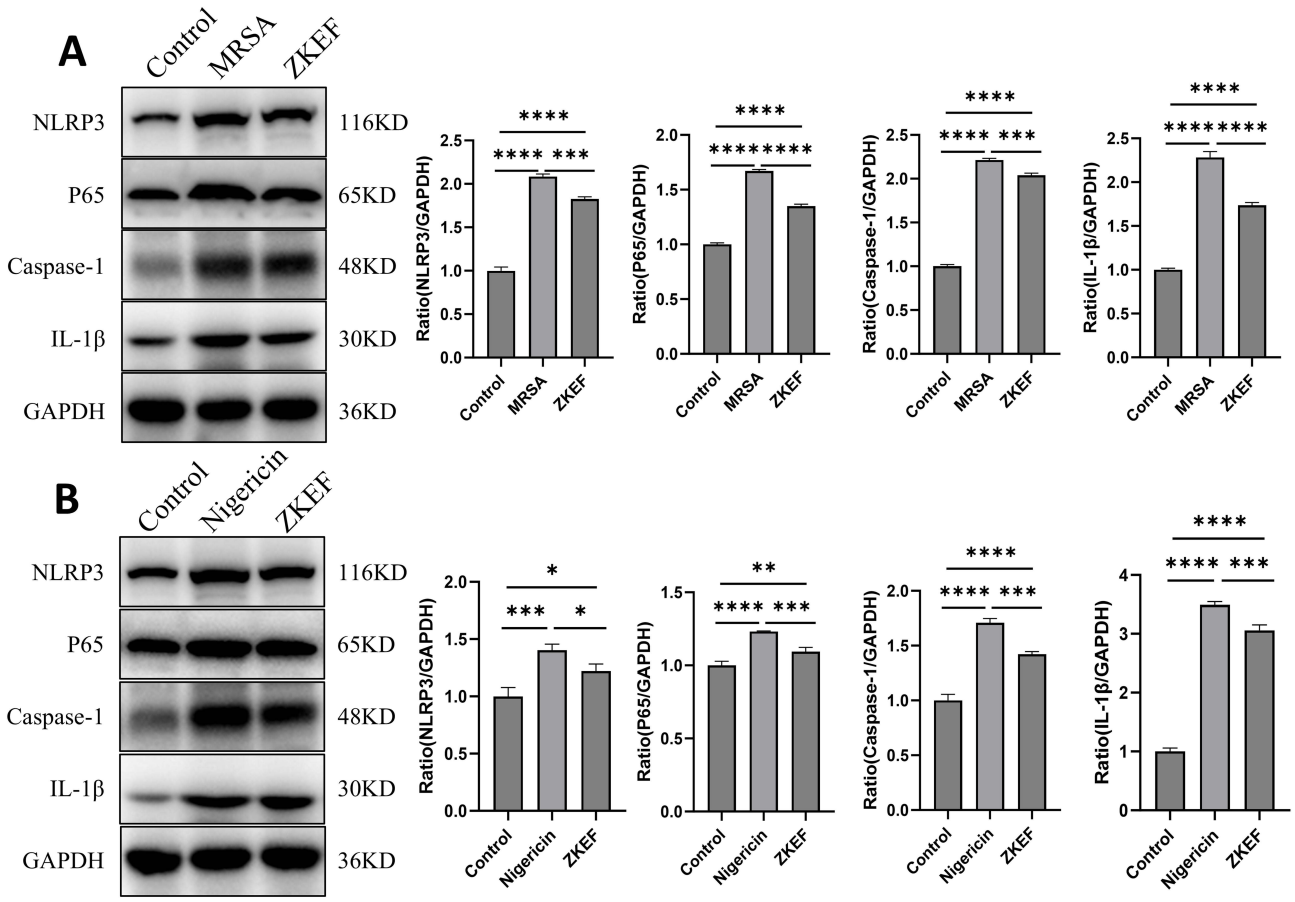

Figure 5 Specificity of Zhike Erfang in Inhibiting NLRP3 Inflammasome Activation in Bone Marrow-Derived Macrophages. (**A**) The expression of NLRP3, NF-κB p65, IL-1β and caspase-1 were measured by Western blot in alveolar macrophages treated with MRSA. (**B**) The expression of NLRP3, NF-κB p65, IL-1β and caspase-1 were measured by Western blot in bone-marrow derived macrophages stimulated with nigericin. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Platycodonis and Glycyrrhizae Radix, for its potential to treat pneumonia caused by MRSA, and explores its underlying mechnism. Through the observation of pathological damage in model tissue, it was found that Zhike Erfang inhibited pneumonia caused by MRSA in a dose-dependent manner, indicating its therapeutic effect on lung injury induced by MRSA.

NLRP3 inflammatory corpuscle is a trimer composed of NLRP3, apoptosis-related spot-like protein (ASC) and caspase-1,[29,] which plays an important role in inflammatory reactions caused by infection and non-infection. There are few studies on the relationship between NLRP3 inflammatory corpuscles and MRSA infection at home and abroad. Muller et al found that peptidoglycan purified from MRSA can activate NLRP3 inflammatory bodies.[30] Macrophages cultured in extracellular vesicles of MRSA undergo inflammasome activation of NLRP3, which depends on the extracellular vesicle medium.[31] In this study, we detected MRSA-induced lung tissue and found that Zhike Erfang inhibited the expression of NLRP3 and caspase-1 in the injured model tissue. NLRP3 inflammatory corpuscles play a role in anti-infective immunity through signal pathways. Previous studies have shown that MRSA skin infection induces the production of IL-1β inflammatory factors by activating inflammatory corpuscles of myeloid cells NLRP3. In this study, we found that Zhike Erfang inhibited the expression of IL-1β and other inflammatory factors in lung tissue injured by MRSA, lung lavage fluid, and macrophages stimulated by MRSA. This shows that Zhike Erfang can inhibit the production of downstream inflammatory factors by inhibiting the activation of NLRP3 inflammatory corpuscles.

Pneumonia caused by MRSA is an acute infectious disease of the lung. Macrophages, as innate immune cells in tissues, are the first line of defense against the invasion of foreign bacteria and participate in the occurrence and maintenance of acute inflammation.[32] Activated macrophages can secrete inflammatory cytokines and mediators (such as TNF-α, IL-1β, iNOS and

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NO), which aggravate the inflammatory reaction and tissue damage.[33] In addition, macrophages express pattern recognition receptors (PRPs), including TLR, to recognize pathogen-related molecular patterns (PAMP) and thus initiate innate immune response. Toll-like receptor 2(TLR2) is considered to be the most important receptor of gram-positive bacteria MRSA.[34] After TLR2 is activated, TRAF6 is recruited, which leads to abnormal downstream signals, which in turn leads to inflammation progress and tissue damage deterioration.[35] In this study, we found that Zhike Erfang inhibited the expression of TRAF6, indicating that Zhike Erfang could ubiquitously degrade TRAF6 after treatment.

Despite significant outcomes, our study acknowledges several limitations. Our study's findings are based on a mouse model, which may not fully capture the complexities of human MRSA-induced pneumonia due to species differences in immune responses. We also focused solely on the MRSA strain USA300, limiting the generalizability across other strains. Additionally, the dose-response relationship was constrained to specific dosages and administration schedules, potentially differing from real-world clinical practices. Moreover, Zhike Erfang is a complex herbal compound, and the study did not distinguish the effects of its individual components, which may vary in their contribution to its therapeutic efficacy.

Conclusion

In conclusion, Zhike Erfang can alleviate pneumonia caused by MRSA by promoting TRAF6 expression and inducing NLRP3 inflammatory body activation. Nevertheless, it remains to be revealed whether the expression of TRAF6 is related to NLRP3 inflammatory corpuscles during the treatment of Zhike Erfang and the time limit for the radical treatment of MRSA lung injury by Zhike Erfang needs to be observed. Moreover, NLRP3 inflammatory bodies also contain caspase-1 and other structural components, and the somatic role, mechanism and signal network in MRSA infection alone or even mixed infection need to be further studied.

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Disclosure

The authors report no conflicts of interest in this work.

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