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Rhein Confers Protection Against Pneumococcal Pneumonia via Immunomodulation of the MAPK/ NF-κB Axis

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ABSTRACT

Background: *Streptococcus pneumoniae* remains a leading cause of community-acquired pneumonia. Severe pulmonary inflammation and subsequent tissue damage are driven significantly by the overexpression of both the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways.

Objective: To investigate the immunomodulatory effects of rhein in a mouse model of Streptococcus pneumoniae-induced pneumonia .

Methods: Forty male C57BL/6 mice were randomly assigned to four experimental groups: healthy control, *S. pneumoniae* infected control, and two rhein-treatment groups (10 mg/kg and 30 mg/kg). Expression levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, transforming growth factor (TGF)-β, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in lung tissues and bronchoalveolar lavage fluid (BALF) were quantified using reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Additionally, ELISA and Western blotting were employed to assess the phosphorylation profiles of MAPK and NF-κB.

Results: Rhein significantly decreased the lung wet/dry weight ratio, reduced bacterial load, and downregulated COX-2 and iNOS expression. Rhein treatment was also associated with reduced pulmonary bacterial load. Cytokine analysis revealed marked reductions in pro-inflammatory mediators (IL-6, IL-1β, TNF-α, and MCP-1) alongside elevated levels of the anti-inflammatory cytokine IL-10 in both lung tissues and BALF. Furthermore, ELISA and Western blotting confirmed that rhein significantly inhibited the phosphorylation of MAPK and NF-κB.

Conclusion: Rhein attenuates *S. pneumoniae*-induced pulmonary inflammation and injury by modulating the MAPK/NF-κB signaling pathways. These findings highlight Rhein as a promising potential therapeutic agent for pneumococcal pneumonia and related inflammatory lung diseases.

Keywords: *S. pneumoniae;* Immunomodulatory; Therapeutic agent; cytokine

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INTRODUCTION

Pneumonia is an acute respiratory infection characterized by inflammation of the lung's alveoli. This inflammation often results inthe accumulation of pus or fluid within the alveolar spaces, thereby compromising physiological gas exchange and and manifesting clinically as cough, fever, dyspnea, and malaise. The severity of pneumonia varies widely, from mild cases to life-threatening ones, with infants, the elderly, and immunocompromised individuals being especially vulnerable (1). Pneumonia is categorized into several distinct types, classified primarily by cause and mode of transmission. Community-acquired pneumonia (CAP) occurs outside of healthcare settings and is commonly caused by bacteria, viruses, and fungi. Hospital-acquired pneumonia (HAP) develops in hospitalized patients, often due to multidrug-resistant bacteria. Ventilator-associated pneumonia (VAP) is a subtype of HAP that occurs in patients receiving mechanical ventilation. Aspiration pneumonia results from inhaling food, liquids, or other substances into the lungs, leading to infection and inflammation. Atypical pneumonia, often caused by pathogens such as *Mycoplasma pneumoniae* and *Legionella pneumophila*, typically presents with milder symptoms but remains a clinically significant health concern (2).

Among the bacterial causes of pneumonia, *Streptococcus pneumoniae (S. pneumoniae)* is the most prevalent and significant pathogen, responsible for a large proportion of bacterial pneumonia cases worldwide (3). *S. pneumoniae* is known for its ability to evade the host immune response, produce virulence factors, and trigger excessive inflammation, often leading to severe lung damage and complications such as bacteremia, pleural effusion, and respiratory failure (4). Despite advances in vaccination and antibiotic therapy, pneumococcal pneumonia remains a leading cause of morbidity and mortality, highlighting the need for novel therapeutic strategies to manage inflammation and improve clinical outcomes (5).

The pathogenesis of *S. pneumoniae*-induced pneumonia involves a complex interplay between the host's immune defenses and bacterial virulence factors (6). Infection triggers a robust immune response, centrally mediated by the activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways. These pathways are essential for host defense, primarily through their regulation of key pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). While these cytokines are crucial for pathogen clearance, their excessive and uncontrolled production can result in a "cytokine storm," leading to severe tissue damage, acute lung injury, and potentially progressing to acute respiratory distress syndrome (ARDS) (7).

Given the dual-edged nature of the inflammatory response in pneumonia, there is growing interest in identifying therapeutic agents that can modulate these pathways to achieve a balance between effective pathogen clearance and minimizing host tissue damage (8). Rhein, a naturally occurring anthraquinone derivative extracted from rhubarb (Rheum species), has emerged as a compound of particular interest in this context. Renowned for its anti-inflammatory, antiproliferative, and antioxidant properties, rhein has been demonstrated to inhibit pivotal inflammatory cascades, including those involving MAPK and NF-κB signaling. Previous investigations have underscored its therapeutic potential across diverse inflammatory pathologies, including malignancies, inflammatory bowel disease, and rheumatoid arthritis. Nevertheless, its efficacy in bacterial pneumonia—specifically *S. pneumoniae*-induced pneumonia—remains largely unexplored (9-11). To address this knowledge gap, the present study sought to evaluate the therapeutic capacity of rhein in a mouse model of *S. pneumoniae*-induced pneumonia. We posited that rhein would modulate

MAPK/NF-κB signaling pathways, thereby attenuating the exuberant i inflammatory response and ameliorating pulmonary injury. A comprehensive series of experiments was conducted to assess the effects of rhein on multiple parameters, including markers of lung injury, inflammatory cytokine production, and overall survival in infected mice.

MATERIALS AND METHODS

Animal model and experimental design

Rhein was purchased from Sigma-Aldrich (Germany), and dosages of 10 mg/kg and 30 mg/kg were selected based on previous studies (12, 13). Male C57BL/6 mice, weighing approximately 20-25 grams and aged 6-8 weeks, were maintained under controlled laboratory conditions (humidity: 50±10%, temperature: 22±2°C, 12-hour light/dark cycle. Animals were acclimatized for one week prior to experimentation, with ad libitum access to a standard laboratory diet and water. All procedures were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and every effort was made to to minimize animal usage and ensure their welfare throughout the study. The experimental protocols received institutional approval and were performed in compliance with relevant national guidelines for the care and use of laboratory animals. The Zhinanzhen Biology Ethics Committee approved this work under approval No. A2024000808.

1. Mice were randomly assigned to four experimental groups (n = 10 per group) as follows: Control Group: Mice received 50 μL of sterile saline intranasally as a negative control.

2. *S. pneumoniae* Group: Mice were intranasally inoculated with 1×10^7 colony-forming units (CFU) of *S. pneumoniae* serotype 3 (ATCC 6303) in 50 μL of sterile phosphate-buffered saline (PBS) to induce pneumonia.

3. *S. pneumoniae* + Low-Dose Rhein Group: Mice were inoculated with *S. pneumoniae* as described above and subsequently received 10 mg/kg of rhein dissolved in 0.5% carboxymethylcellulose (CMC) via orogastric gavage once daily for 7 days.

S. pneumoniae + High-Dose Rhein Group: Mice were inoculated with *S. pneumoniae* and treated with 30 mg/kg of rhein using the same administration route and schedule as the low-dose group. Randomization was performed using a computer-generated sequence and investigators responsible for sample collection and outcome assessments were blinded to the group allocations throughout the experiment to minimize bias. Sample size (n = 10 per group) was determined based on previously published studies utilizing S. pneumoniae-induced pneumonia models, which demonstrated significant biological effects with 8–10 animals per group when assessing inflammatory responses, cytokine levels, and lung injury indices (14). A post-hoc power analysis was conducted using GraphPad Prism (v9.0) based on observed effect sizes for cytokine concentrations and lung injury scores. The achieved statistical power (1–β) ranged from 0.82 to 0.90 at α = 0.05, confirming that the study possessed sufficient sensitivity to detect the observed group differences.

Induction of pneumonia

Pneumonia was induced in the designated groups via intranasal administration of *S. pneumoniae* (ATCC 6303 serotype 3 [ST 3]) at a dose of 1×10^7 CFU per mouse, under light anesthesia using 2-3% isoflurane (Baxter Healthcare, NDC: 10019-360-60). The inoculation procedures were performed within a biosafety cabinet under aseptic conditions to preclude contamination. The bacterial suspension was freshly prepared from an overnight culture in Todd-Hewitt broth, and the concentration was adjusted spectrophotometrically (OD_{600} = 0.1 corresponded to approximately 1×10^8 CFU/mL) (14).

Throughout the study, mice were closely monitored for clinical signs of pneumonia, body weight changes, and overall health status. Any animal exhibiting severe symptomatology or weight loss exceeding 20% of initial body weight was humanely euthanized in accordance with the ethical guidelines (15). At the experimental endpoint, mice were sacrificed via anesthetic overdose. Lung tissues were subsequently harvested for comprehensive analysis, including Western blotting, enzyme-linked immunosorbent assay (ELISA), and reverse transcription-polymerase chain reaction (RT-PCR), to evaluate the effects of rhein on inflammatory pathways and pulmonary tissue integrity.

Assessment of lung injury

Lung injury was quantitatively evaluated by determining the lung wet/dry (W/D) weight ratio and pulmonary bacterial load, as described below.:

Lung wet/dry ratio: Pulmonary edema was assessed via the lung wet/dry weight ratio. Twenty-four hours Pulmonary edema was assessed via the the final rhein administration, mice were euthanized by an intraperitoneal injection of an overdose of pentobarbital (100 mg/kg). Lungs were immediately excised, and the wet weight was recorded. Tissues were then desiccated in a drying oven at 60°C for 72 hours to obtain the dry weight, after which the W/D ratio was then calculated.

Bacterial load: Lung tissues were homogenized in sterile PBS using a mechanical homogenizer (TissueRuptor, Qiagen, Germany). Serial dilutions of the homogenates were subsequently plated onto 5% sheep blood agar medium (HyMedia-India) and incubated for 18-24 hours at 37°C in a 5% CO_2 atmosphere. Bacterial colonies were enumerated by counting CFUs.

Cytokine measurement

Concentrations of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, (R&D Systems, nos.: M6000B, MLB00C, and MTA00B, respectively), anti-inflammatory cytokines IL-10, TGF-β and MCP-1 (R&D Systems, catalog nos.: M1000B-1, DY1679-05, and MJE00B, respectively), cyclooxygenase-2 (COX-2,catalog no. A312741, Antibodies, UK), and inducible nitric oxide synthase (iNOS,catalog no. A310723, Antibodies, UK) in BALF were quantified using commercially available ELISA kits, following the manufacturer's instructions. BALF was obtained by washing the lungs with 1 mL of sterile PBS, and cell-free supernatants were collected by lavaging the lungs with 1 mL of sterile PBS, and cell-free supernatants were isolated via centrifugation at 400×g for 10 minutes at 4°C.

Gene Expression Analysis by qPCR

Messenger RNA (mRNA) expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), anti-inflammatory cytokines (IL-10, TGF-β and MCP-1), COX-2, and iNOS were quantified via qRT-PCR. Total RNA was first extracted from lung homogenates using TRIzol reagent. Following isolation, RNA concentration and purity were assessed by spectrophotometry.

For complementary DNA (cDNA) synthesis, 1 µg of total RNA from each sample was reverse-transcribed. using a Reverse Transcriptase Kit (BioFACT, Korea), strictly adhering to the manufacturer's protocol. The resulting cDNA was then subjected to qPCR using SYBR Green master mix (BioFACT, Korea) with gene-specific primers for each target and the housekeeping gene GAPDH as an internal control.

The qPCR amplification protocol consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method, with Ct values normalized to GAPDH.

Western blot analysis

The phosphorylation status of MAPKs (p38, JNK, and ERK) and of NF-κB p65 was evaluated using western blot analysis. Lung

tissue lysates were prepared by homogenizing samples in RIPA buffer supplemented with protease and phosphatase inhibitors. Total protein concentration were determined using a BCA assay kit (Sigma-Aldrich, Germany). Proteins (30 μg per lane) were resolved by SDS-PAGE on 10% polyacrylamide gels, and subsequently electrotransferred onto PVDF membranes (Millipore, USA). Following a 1-hour blocking step in 5% non-fat dry milk prepared in TBST, membranes were incubated overnight at 4 °C with primary antibodies (Cell Signaling Technology, USA) directed against phospho-p38, phospho-JNK, phospho-ERK1/2 and phospho-NF-κB p65 (all at 1:1000 dilution). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific, 32106, USA) and imaged with a ChemiDoc MP imaging system (Bio-Rad, USA) (16).

Measurement of phosphorylated MAPK and NF-κB p65 by ELISA

To quantitatively evaluate the activation of key inflammatory signaling pathways, the phosphorylation levels of p38, JNK, ERK1/2, and NF-κB p65 were measured in homogenized lung tissue samples using commercially available sandwich ELISA kits (MBS3805594, MyBioSource, USA; DYC1387B-2, R&DSystem, USA; EMS2ERKP, Invitrogen, USA; MBS009329, MyBioSource, USA, respectively) following the manufacturer's protocol. Protein supernatants obtained from lung tissue homogenates (as described in Section 2.6) were utilized. Absorbance values were measured at 450 nm using a microplate reader.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (v9.0, GraphPad Software, CA, USA). Data are presented as mean ± standard deviation (SD). For comparisons among multiple groups, one-way analysis of variance (ANOVA) was employed, followed by Tukey's post hoc test for multiple comparisons. For non-parametric datasets, the Kruskal-Wallis H test was applied, with Dunn's test for post hoc analysis. Survival data were evaluated using Kaplan-Meier survival curves, and differences between groups were assessed using the log-rank (Mantel-Cox) test. A p-value of <0.05 was considered statistically significant across all analyses.

RESULTS

Analysis of lung injury

Rhein treatment significantly attenuated lung injury in the mouse model, as evidenced by a marked reduction in the lung W/D weight ratio. The W/D ratio was significantly lower in both the low-dose (5.25 ± 0.39) and high-dose (5.18 ± 0.23) rhein-treated groups compared to the *S. pneumoniae* group (6.21 ± 0.34), indicating diminished pulmonary edema and fluid accumulation. One-way ANOVA followed by Tukey's post hoc test revealed that the differences between both rhein-treated groups and the *S. pneumoniae* group were statistically significant ($p < 0.001$, Figure 1A).

Analysis of bacterial load

Rhein treatment significantly reduced pulmonary bacterial loads in infected mice. Both low-dose and high-dose rhein groups exhibited markedly lower bacterial counts compared to the S. pneumoniae group ($p < 0.001$, Figure 1B). While this observation suggests improved bacterial control, the underlying mechanism requires further investigation.

Analysis of cytokine levels in lung tissue

Using RT-PCR to assess gene expression in lung tissue, we found that rhein treatment dramatically altered the expression of key inflammatory mediators. The mRNA expression levels of the pro-inflammatory

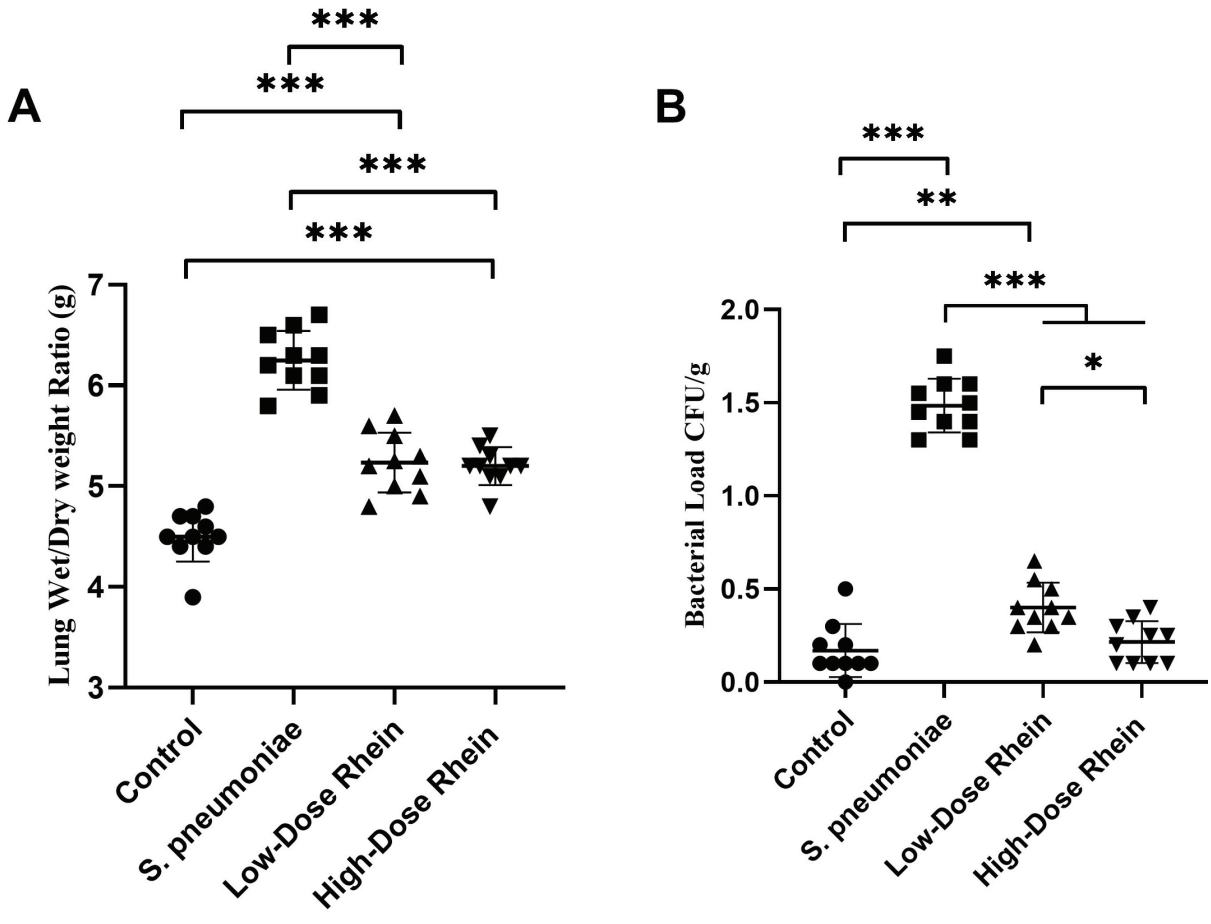

Figure 1. Assessment of lung injury in the control, *S. pneumoniae* infected, and rhein-treated groups. A. Lung wet/dry weight ratio, reflecting pulmonary edema severity. B. Pulmonary bacterial load, quantified as colony-forming units (CFUs). Data are presented as mean ± SD (n=10 per group). Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, with $p < 0.05$ considered significant. Each experiment was performed in triplicate. *p<0.05, **p<0.01, ***p<0.001; ns: not significant.

cytokines TNF-α, IL-1β, and IL-6, were all significantly upregulated in the untreated S. pneumoniae group compared to healthy controls. Treatment with rhein significantly downregulated these genes in a dose-dependent manner, with the high-dose group demonstrating the largest reduction.

Rhein treatment also significantly altered the expression of anti-inflammatory and regulatory genes. The expression of IL-10 and TGF-β, two anti-inflammatory cytokines, were markedly enhanced in high-dose rhein groups compared to the infected, untreated group. Conversely, transcription of MCP-1, which was elevated upon infection, was significantly downregulated by rhein treatment in a dose-dependent manner.

We also examined the expression of the inflammatory enzymes COX-2 and iNOS. COX-2. Both genes were highly elevated in the S. pneumoniae group, but this elevation was substantially diminished with rhein treatment, particularly at the highest dose (Figure 2).

In summary, rhein appears to act at the molecular level to both inhibit the pro-inflammatory cascade and restore a more balanced immune response by promoting IL-10 expression and dampening transcription of TGF-β, MCP-1, COX-2, and iNOS in the lungs of infected mice.

Analysis of cytokine levels in BALF

ELISA findings demonstrated that rhein treatment significantly altered the inflammatory profile of the BALF collected from infected mice.

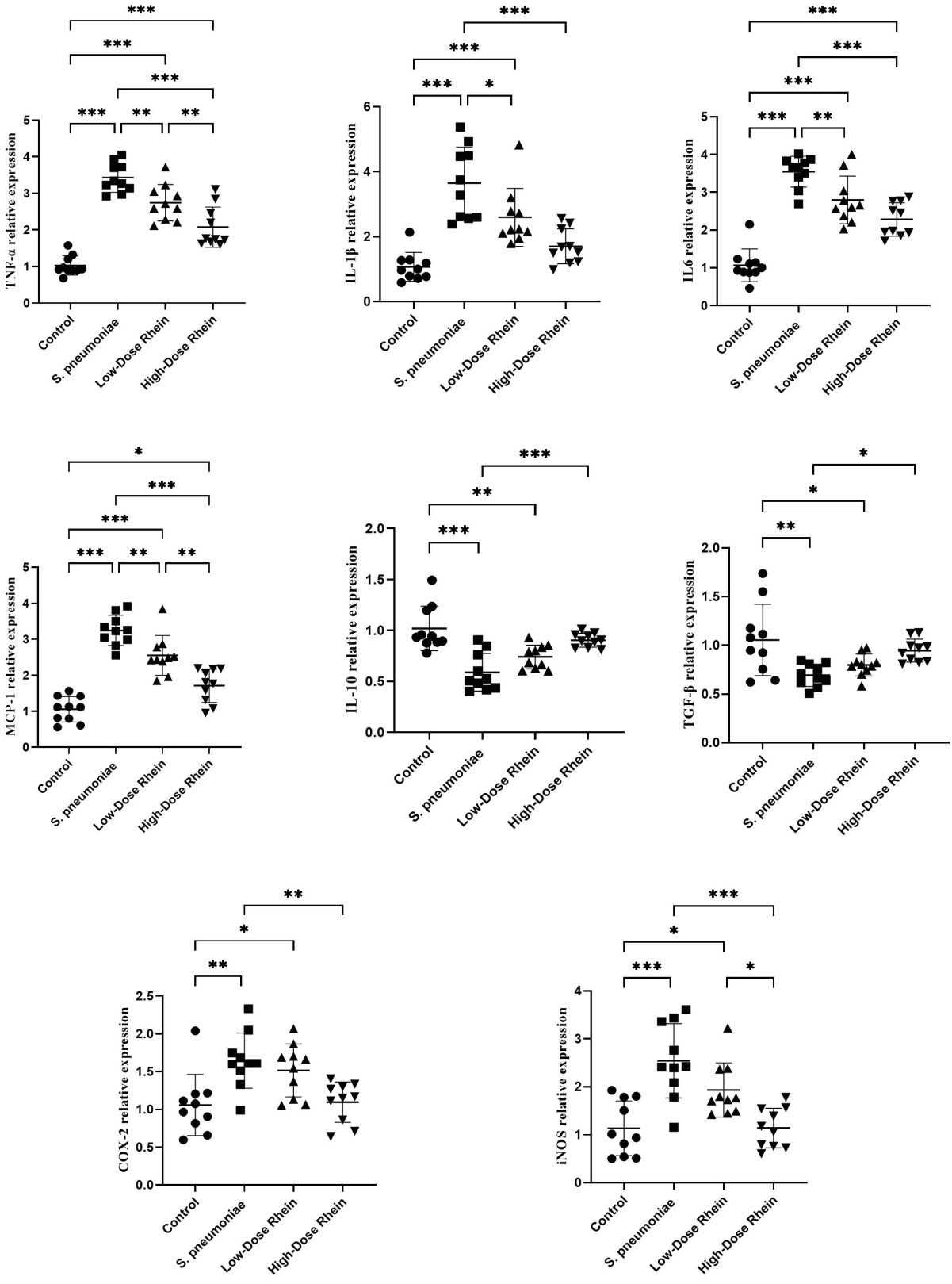

Figure 2. Rhein treatment attenuates pro-inflammatory cytokine expression in lung tissue in a murine model of S. pneumoniae infection. mRNA expression levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were measured in lung tissue homogenates by quantitative Real-time PCR. Data demonstrate a significant, dose-dependent reduction in cytokine levels in rhein-treated mice compared to the untreated infected group. Values are presented as mean ± SD (n=10 per group). Statistical significance was defined as $p < 0.05$. Each experiment was performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

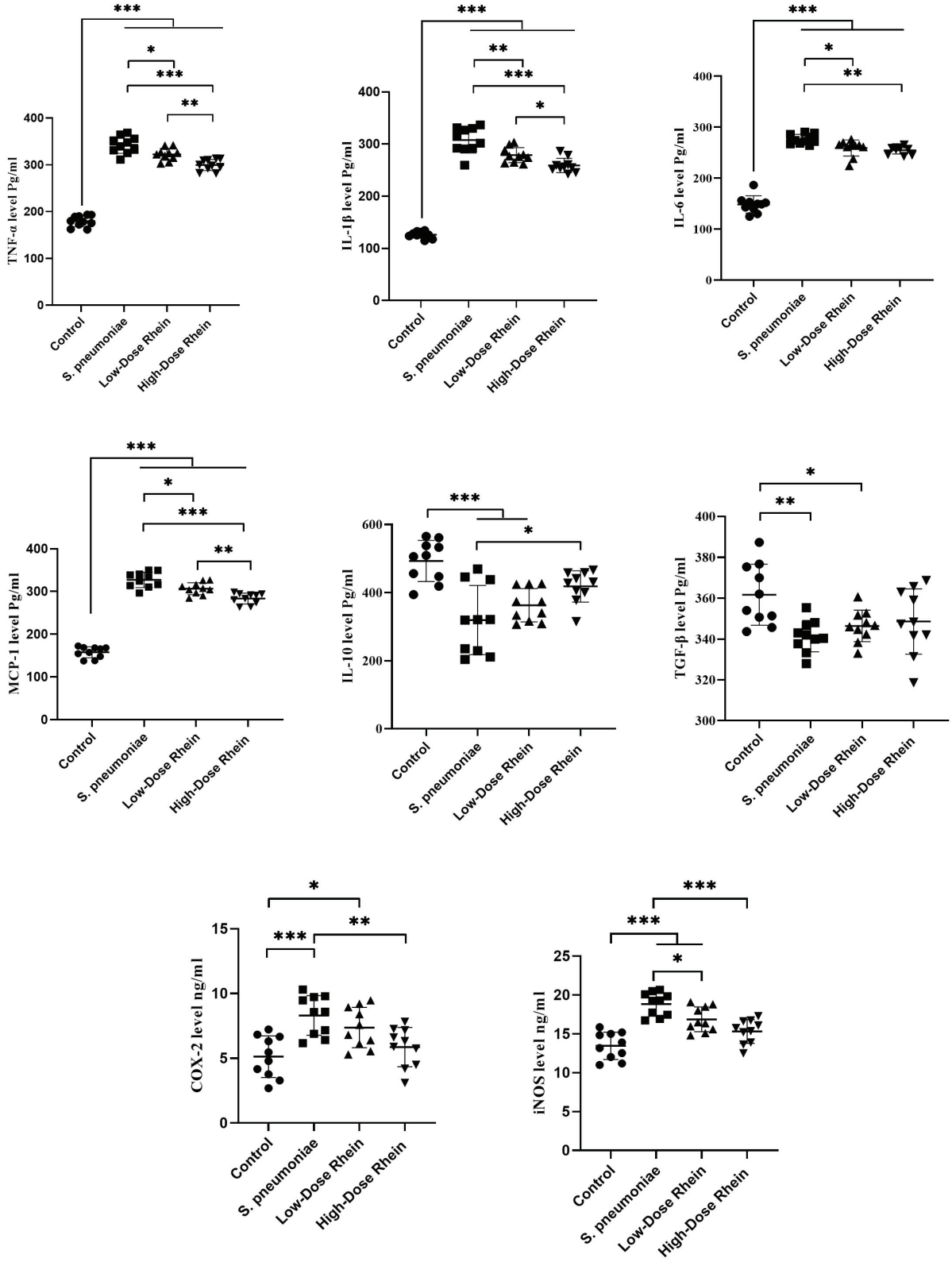

Figure 3. Rhein treatment modulates cytokine and inflammatory mediator levels in bronchoalveolar lavage fluid (BALF) in a murine model of S. pneumoniae-induced pneumonia. Concentrations of TNF-α, IL-1β, IL-6, IL-10, TGF-β, MCP-1, COX-2, and iNOS in BALF were determined by ELISA. Rhein treatment significantly and dose-dependently reduced pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), the chemokine MCP-1, and the inflammatory enzymes COX-2 and iNOS, while significantly increasing the anti-inflammatory cytokine IL-10. Values are presented as mean ± SD (n=10 per group). Statistical significance was defined as $p < 0.05$. Each experiment was performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

As anticipated, concentrations of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and MCP-1 were markedly elevated in the untreated S. pneumoniae group compared to healthy controls. Treatment with rhein significantly reduced the concentrations of these cytokines in a dose-dependent manner, with the high-dose group achieving the greatest reduction.

We also examined anti-inflammatory and regulatory mediators to further characterize the immunomodulating properties of rhein. Concentrations of the anti-inflammatory cytokine IL-10 were significantly increased in both rhein-treated groups compared to the infected untreated group. In contrast, the pro-inflammatory chemokine MCP-1 was significantly reduced by rhein treatment. Additionally, TGF-β levels showed no significant changes in rhein-treated groups relative to the infected untreated group, suggesting that rhein exerts a more sophisticated regulatory role beyond simple immunosuppression.

We further evaluated the expression of key inflammatory enzymes COX-2 and iNOS. Both were significantly upregulated in the S. pneumoniae group, and this upregulation was effectively inhibited by rhein treatment, with the highest dose again producing the most potent effect (Figure 3).

Collectively, these data indicate that rhein treatment not only suppresses the pro-inflammatory cytokine storm but also promotes a more balanced immune response by enhancing IL-10 while concurrently inhibiting MCP-1, COX-2, and iNOS expression in the airways of infected mice.

Analysis of MAPK/NF-κB signaling

ELISA and Western blot analysis of lung tissue samples demonstrated the inhibitory effect of rhein on phosphorylated MAPKs (p38, JNK, ERK) and NF-κB p65. The expression levels of these signaling proteins varied significantly among the experimental groups, highlighting the dose-dependent effect of rhein on inflammatory pathways.

In the *S. pneumoniae* group, there was a marked increase in the phosphorylation of p38, JNK, ERK, and NF-κB p65, indicating strong activation of the MAPK/NF-κB pathway in response to infection. However, treatment with rhein resulted in a notable reduction in the phosphorylation levels of these proteins, with the extent of inhibition depending on the administered dose. The high-dose rhein group exhibited substantial suppression of MAPK/NF-κB signaling (Figure 4), suggesting a potent anti-inflammatory effect that likely contributed to the mitigation of lung tissue inflammation.

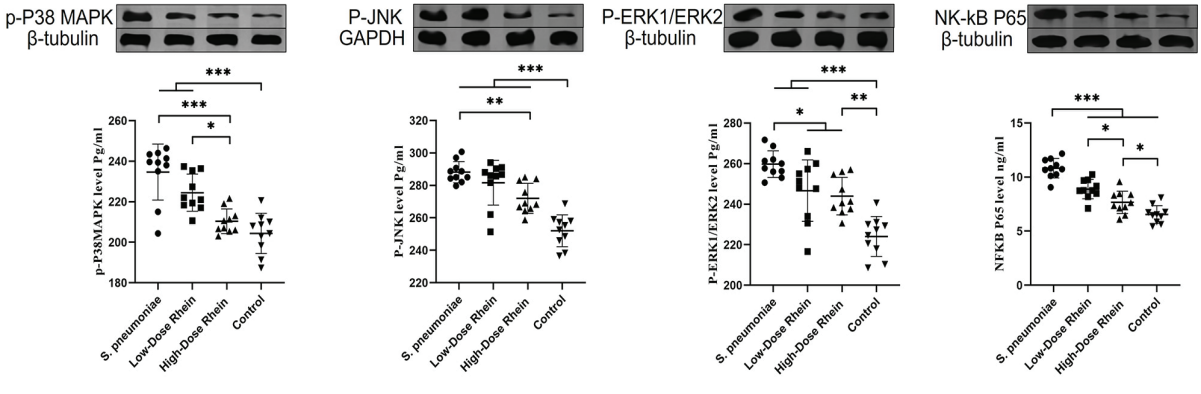

Figure 4. Rhein inhibits the phosphorylation of MAPK and NF-κB signaling proteins in lung tissue. ELISA and Western blot analysis demonstrate the expression levels of phosphorylated MAPK proteins (p38, JNK, ERK) and NF-κB p65 in lung homogenates from each experimental group. Data show a dose-dependent reduction in phosphorylation levels following rhein treatment. Values are presented as mean ± SD (n=10 per group). Statistical significance was defined as $p < 0.05$. Each experiment was performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MAPK, Mitogen-Activated Protein Kinase; JNK, c-Jun N-terminal kinase; ERK, Extracellular Signal-Regulated Kinase; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells.

The low-dose rhein group also demonstrated an inhibitory effect, though to a lesser extent.

The control group, which was neither infected with S. pneumoniae nor treated with rhein, maintained baseline expression levels of MAPKs and NF-κB p65, further confirming that the observed changes in phosphorylation levels were directly associated with infection and treatment. The vehicle-treated group (infected but receiving no rhein) exhibited elevated phosphorylation levels similar to the *S. pneumoniae* group, reinforcing that rhein specifically modulates these pathways.

DISCUSSION

The pathogenesis of *S. pneumoniae*-induced pneumonia involves a complex interplay between the host immune response and bacterial virulence factors (17). A key component of this response is the activation of the MAPK and NF-κB signaling pathways, which play central roles in regulating the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6—cytokines that are crucial for effective innate immune defense and pathogen clearance. However, excessive or dysregulated activation of these pathways can result in an exaggerated inflammatory response, leading to alveolar–capillary barrier disruption, lung tissue injury, and, in severe cases, acute respiratory distress syndrome (ARDS) (18).

Given the dual role of inflammation in both host defense and tissue injury during pneumonia, identifying therapeutic strategies that can effectively modulate these inflammatory pathways is crucial. Rhein, a natural anthraquinone isolated from rhubarb, exhibits well-documented anti-inflammatory properties. Its therapeutic potential is largely attributed to its ability to inhibit key inflammatory signaling pathways, including the MAPK and NF-κB pathways (19). Although previous studies have demonstrated the anti-inflammatory efficacy of rhein in a variety of inflammatory diseases, its therapeutic potential in bacterial pneumonia, particularly *S. pneumoniae*-induced pneumonia, remains insufficiently investigated. Therefore, in the present study, we evaluated the effects of rhein on the MAPKs and the NF-κB signaling pathway and assessed its therapeutic potential in a mouse model of *S. pneumoniae*-induced pneumonia (20, 21).

The results of the present study showed that the lung wet/dry weight ratio, a widely used indicator of pulmonary edema, was significantly lower in the rhein-treated groups compared to the untreated *S. pneumoniae* group. This reduction suggests that rhein treatment effectively reduces lung injury by limiting pulmonary fluid accumulation. The decreased lung wet/dry weight ratio indicates a protective effect of rhein against *S. pneumoniae*-induced lung injury, likely through its anti-inflammatory activity. These findings are consistent with previous studies demonstrating the protective effects of rhein against tissue edema and inflammatory injury in various disease models. For example, Zhao et al. (2023) reported that rhein treatment attenuated the inflammatory response associated with cardiomyopathy in both in vivo and in vitro models (22).

The present study demonstrates that rhein treatment markedly modulates the pulmonary inflammatory response during Streptococcus pneumoniae infection. This effect was evidenced by RT-PCR and ELISA analyses, which showed a dose-dependent reduction in the expression and production of the pro-inflammatory mediators TNF-α, IL-1β, IL-6, and MCP-1, accompanied by an increase in the anti-inflammatory cytokine IL-10. Collectively, these findings suggest that rhein exerts immunomodulatory effect that contribute to the restoration of immune homeostasis without completely suppressing host immune function.

Infection with S. pneumoniae activates the innate immune system, triggering the production of pro-inflammatory cytokines primarily through the activation of the NF-κB

and MAPK signaling pathways. The elevated levels of TNF-α, IL-1β, and IL-6 observed in the infected mice are consistent with the 'cytokine storm' associated with severe bacterial pneumonia, which contributes to increased alveolar–capillary permeability, pulmonary edema, and lung tissue injury (4, 18). Therefore, the ability of rhein to reduce the levels of these pro-inflammatory cytokines suggests that it may attenuate the exaggerated inflammatory response underlying S. pneumoniae-induced lung injury. Consistent with these findings, previous studies have reported that rhein suppresses the production of these cytokines by inhibiting NF-κB nuclear translocation and MAPK phosphorylation (9, 13).

The observed decrease in MCP-1 (CCL2) mRNA and protein levels provides additional support for rhein's regulatory capacity in leukocyte recruitment. MCP-1 is a chemokine critical for monocyte and macrophage trafficking; however, its overexpression can promote inflammatory infiltration and exacerbate pulmonary pathology (23). By reducing MCP-1 expression, rhein may help mitigate excessive immune cell infiltration, thereby preserving alveolar integrity and improving gas exchange.

The increase in IL-10 expression and concentration observed in both lung tissue and BALF provides additional evidence of rhein's immunoregulatory mechanism. IL-10 is a pivotal anti-inflammatory cytokine that inhibits pro-inflammatory cytokine synthesis, thereby attenuating inflammation and limiting tissue injury (24). The dose-dependent enhancement of IL-10 observed in this study is consistent with the findings of Li et al. (2023), who reported that rhein increased IL-10 production in a lipopolysaccharide (LPS)-induced acute lung injury model, resulting in improved pulmonary function (25). Notably, rhein had no significant effect on TGF-β signaling, suggesting that its anti-inflammatory action is selective and not associated with fibrosis-promoting pathways—a beneficial quality compared to corticosteroids or other broad-spectrum immunosuppressants (26).

The reduction of COX-2 and iNOS production in lung tissue and BALF also highlights rhein's effects on downstream mediators of inflammation. Both enzymes are upregulated in response to bacterial infection and exacerbate oxidative and nitrosative stress, and contributing to lung injury (27). The inhibition of COX-2 and iNOS by rhein has been demonstrated in macrophage and epithelial cell models and occurs largely occurring through the suppression of NF-κB-mediated gene expression (11, 16). Through downregulation of these mediators, rhein may reduce inflammatory tissue damage and promote the resolution of injury and infection-associated pathology.

ELISA and Western blot analysis revealed that rhein treatment significantly suppressed the phosphorylation of MAPKs (p38, JNK, ERK) and NF-κB p65 in lung tissues compared to the *S. pneumoniae*-infected group. This indicates that the anti-inflammatory mechanism of rhein is mediated through the modulation of these critical signaling pathways, which are central to coordinating the inflammatory response. These findings align with previous research, such as that of Henamayee et al. (2020), who reported that rhein inhibited MAPK/NF-κB pathways in a rat model of rheumatoid arthritis, resulting in reduced inflammation and joint damage (9). Collectively, these results underscore rhein's therapeutic potential in controlling inflammation through the modulation of key signaling pathways.

Previous studies have demonstrated that the inhibition of MAPK/NF-κB signaling pathways is strongly associated with reduced inflammation and improved disease outcomes in various inflammatory conditions. For instance, Liu et al. (2021) reported that suppression of these pathways led to decreased cytokine production and tissue damage in a rheumatoid arthritis model, while Li et al. (2018) showed similar effects in an acute lung injury model (28, 29). Additionally, studies on

other anti-inflammatory compounds, such as curcumin and resveratrol, have established a clear link between MAPK/NF-κB inhibition and reduced inflammation-related pathology. In our study, the significant reduction in pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) further supports the premise that MAPK/NF-κB pathway inhibition plays a crucial role in mediating rhein's protective effects. However, we acknowledge that direct causality was not established, as no conditional knockout models or pathway-specific inhibitors were employed. Furthermore, while downstream mediators such as COX-2 and iNOS were not analyzed, the consistency of our findings with prior research strengthens the hypothesis that rhein modulates inflammation through MAPK/NF-κB suppression.

The significant reduction in bacterial load observed in the high-dose rhein group suggests that rhein may possess direct antibacterial properties in addition to its well-characterized immunomodulatory effects. While our study primarily focused on the suppression of inflammatory pathways through MAPK/NF-κB inhibition, the extent of bacterial clearance raises an important question: Does rhein exert direct antimicrobial activity against S. pneumoniae, or is this effect solely a consequence of enhanced host immunity? Previous studies have documented the antibacterial properties of anthraquinones, including rhein, against various bacterial pathogens. For instance, rhein has been shown to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* by disrupting bacterial cell membranes and interfering with essential metabolic pathways (30, 31). Additionally, Rhein has been reported to exhibit antibacterial activity against *Staphylococcus aureus* (32). However, data on its direct effects against *S. pneumoniae* remain limited. Although Rhein treatment significantly reduced lung bacterial burden, it remains uncertain whether this reflects a direct antibacterial effect or an indirect consequence of improved immune clearance. Future studies employing minimum inhibitory concentration (MIC) assays and macrophage killing assays will be necessary to distinguish between direct antimicrobial activity and host-mediated clearance.

The potential dual mechanism of rhein—combining direct antimicrobial activity combined with immunomodulation—could offer a significant advantage over conventional therapies, particularly in an era of increasing antibiotic resistance. If rhein exhibits bacteriostatic or bactericidal effects, it could reduce bacterial burden while simultaneously mitigating excessive inflammation, thereby addressing both pathogen clearance and immune-driven tissue damage. This mechanism is reminiscent of other host-directed therapies, such as macrolides, which not only suppress bacterial growth but also modulate immune responses (33). Given this limitation, we suggest that future investigations should focus on determining whether rhein inhibits *S. pneumoniae* growth through direct interaction with bacterial targets, such as cell wall synthesis, DNA replication, or metabolic pathways.

This finding is consistent with previous studies where rhein reduced tissue edema and damage in other inflammatory models, such as rheumatoid arthritis and nephritis. However, our study is novel in that it applies these findings to a bacterial pneumonia model, specifically highlighting rhein's potential in reducing lung injury associated with excessive inflammation. This suggests that rhein's protective effects on lung tissue may be mediated through its ability to modulate inflammatory signaling pathways, thereby preventing the overwhelming immune response that leads to fluid accumulation and tissue damage.

While the present findings highlight rhein's anti-inflammatory and lung-protective effects in a murine model of *S. pneumoniae*-induced pneumonia, these results remain preclinical and should be interpreted as exploratory. To date, available pharmacokinetic studies indicate that rhein exhibits moderate oral

bioavailability (approximately 20–30%), undergoes hepatic metabolism primarily via glucuronidation and sulfation, and is excreted mainly in the urine (33). Reported toxicity studies in rodents have shown that rhein is generally well tolerated at doses up to 50 mg/kg/day, although chronic administration at higher concentrations may cause mild hepatic or renal alterations (10, 11). However, these pharmacological parameters were not evaluated in the current study, and further research is required to define optimal dosing, distribution kinetics, and safety in pulmonary tissue contexts. Consequently, the present work should be viewed as a mechanistic proof-of-concept rather than a definitive therapeutic evaluation.

While our findings offer valuable insights into the therapeutic potential of rhein for pneumonia, several study limitations should be acknowledged. The absence of an antibiotic-treated control group limits the ability to compare rhein's efficacy with standard treatments; however, due to experimental constraints, this was not feasible. Future studies should incorporate an antibiotic-treated group to better contextualize rhein's effectiveness. Additionally, although key inflammatory markers and signaling pathways were analyzed, further mechanistic investigations—such as transcriptomic and proteomic analyses—could provide a deeper understanding of rhein's mode of action, particularly its effects on immune responses and bacterial clearance. The use of a murine model, despite its translational significance, does not fully replicate human pneumonia, necessitating validation in human-relevant systems such as lung organoids, ex vivo lung tissue cultures, or clinical trials. Moreover, the pharmacokinetics and bioavailability of rhein in lung tissue were not extensively explored, highlighting the need for future studies on its distribution, metabolism, clearance, and optimal dosing regimen. Another important aspect not investigated is the temporal effect of rhein administration, particularly early versus late intervention, which is crucial for determining its optimal therapeutic window. While the inhibition of MAPK/NF-κB phosphorylation strongly suggests rhein's mechanistic involvement, we acknowledge that pathway causality cannot be definitively established without specific inhibitors or knockout models. Follow-up studies using NF-κB p65 or p38 MAPK-deficient mice, or pharmacological inhibitors, are planned to confirm this causal relationship. Although the sample size of ten animals per group was sufficient to detect the main effects in cytokine expression and lung injury indices, it may be underpowered to identify more subtle biological differences. The use of larger cohorts in future studies will help to increase statistical precision and minimize the risk of Type II errors. This study adhered to the 3Rs principle and followed ethical approval limits on animal use. The present study did not include a vehicle-only group (CMC without rhein) or a positive control (dexamethasone or antibiotic-treated group). The CMC vehicle used for rhein suspension is an inert, non-immunogenic carrier that does not independently alter inflammatory or signaling outcomes, as confirmed in pilot observations and prior studies. The focus of this investigation was to establish rhein's mechanistic influence on inflammatory signaling rather than its comparative therapeutic potency. Future confirmatory studies will incorporate both vehicle and positive control groups to provide a complete pharmacodynamic assessment.

CONCLUSION

In conclusion, our study provides evidence that rhein effectively reduces lung injury, bacterial load, and pro-inflammatory cytokine production in a mouse model of *S. pneumoniae*-induced pneumonia. The data support a mechanism whereby rhein alleviates disease pathology through targeted inhibition of the MAPK and NF-κB inflammatory signaling pathways. These findings

are consistent with rhein's known anti-inflammatory and antimicrobial properties while extending its therapeutic potential to to the context of bacterial pneumonia. Importantly, while rhein enhanced TGF-β gene transcription, it did not affect TGF-β protein levels, suggesting that its anti-inflammatory effects are not associated with fibrotic risks. Collectively, our results suggest that rhein may serve as a promising lead compound with immunomodulatory potential against inflammatory lung injury. However, given the preclinical nature of this study, further investigations into pharmacokinetics, toxicity, dosing optimization, and clinical safety are necessary before rhein can be considered a viable therapeutic candidate.

COMPETING INTEREST

The authors declare that they have no conflict of interest.

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