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Calycosin ameliorates airway inflammatory injury induced by respiratory syncytial virus *via* inhibiting NLRP3 inflammasome activation in mice

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Abstract. Respiratory syncytial virus (RSV) is a predominant pathogen inducing respiratory tract infections among infants and elderly. Calycosin exhibits antiviral properties, yet its efficacy against RSV has not been elucidated. In this study, cyclophosphamide was administered to induce immune deficiency in adult mice, following which RSV was introduced *via* the intranasal route to establish an RSV infection model in mice. The viral load in lung tissue, airway resistance, inflammatory cell quantity and pro-inflammatory factor levels within bronchoalveolar lavage fluid (BALF) were quantified. Pathological changes were examined and the NOD-like receptor protein 3 (NLRP3) inflammasome activation status was assessed in lung tissue. The results demonstrated that Calycosin treatment markedly decreased the RSV viral load inside lung tissue, decreased inflammatory factor quantity and pro-inflammatory factor levels within BALF, and alleviated airway resistance, inflammatory injury, as well as goblet cell hyperplasia of mouse lung tissue. Notably, Calycosin significantly suppressed NLRP3 inflammasome activation inside RSV-infected mouse lung tissue. However, co-treatment with Nigericin diminished the protective effects of Calycosin on airway inflammatory injury in RSV-infected mice. These findings indicate that Calycosin alleviates airway injury induced by RSV infection, and the underlying mechanism is associated with the inhibition of the inflammatory response mediated by NLRP3 inflammasome activation.

Keywords: Respiratory syncytial virus — Airway inflammatory — Calycosin — NLRP3 inflammasome

Introduction

Respiratory syncytial virus (RSV) is a predominant pathogen inducing lower respiratory tract infections among babies, the elderly, as well as immunocompromised people (Nam and Ison 2021). RSV infection shows several major pathological manifestations, encompassing acute bronchi-

olitis, respiratory mucosal lesions, alongside pneumonia (Borchers et al. 2013). In China, 16.8% of cases with acute respiratory tract infections tested positive for RSV, with the highest detection rate observed in children (25.7%) (Li et al. 2021). RSV infection poses a significant threat to public health and imposes a substantial economic burden. However, current therapeutic options for RSV infection are limited, as broad spectrum antiviral agents exhibit restricted efficacy and potential adverse effects, while immunomodulatory therapies lack specificity (Walter and Munoz 2025). Consequently, it is urgently needed to identify strategies with high safety and efficacy for combating RSV infection.

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RSV primarily infects respiratory epithelial cells, binding to host cell surface receptors *via* adhesion proteins and entering cells through fusion protein-mediated mechanisms. Subsequently, RSV exploits the host's immune system to elicit a robust inflammatory response, resulting in airway inflammation and tissue damage (González et al. 2012). During RSV infection, inflammasomes are activated to amplify inflammatory response, as a result, proinflammatory factors are released and pyroptosis is induced (Shen et al. 2019; Bedient et al. 2020). As the multiprotein complex, inflammasome contains one sensor protein (e.g., NOD-like receptor protein 3, NLRP3), one effector protein (Caspase-1), together with one adaptor protein (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC). RSV infection can trigger NLRP3 inflammasome activation through regulating signaling pathways or enhancing reactive oxygen species production as well as potassium efflux (Segovia et al. 2012). Therefore, the inhibition of the inflammatory response mediated by the NLRP3 inflammasome can effectively alleviate tissue damage induced by excessive inflammation resulting from RSV infection (Shen et al. 2018; Cheng et al. 2023).

Calycosin is a naturally occurring isoflavone compound primarily derived from the leguminous plant *Astragalus membranaceus*. It exhibits a variety of biological activities, including anti-inflammatory, anti-oxidative, anti-tumoral, neuroprotective, and immune-regulatory effects (Guo et al. 2012; Qu et al. 2022; Tian J et al. 2022; Ding et al. 2023). Network pharmacology analysis identified NLRP3, IL-1 β , and IL-18 as the core targets of Calycosin regulation (Yuan et al. 2025). Calycosin has been shown to mitigate tissue inflammatory injury through the inhibition of NLRP3 inflammasome activation (Zhang et al. 2022; Liao et al. 2024). However, the anti-RSV pharmacological activity of Calycosin remains unclear. Consequently, we propose the hypothesis that Calycosin may alleviate respiratory tract injury induced by RSV infection *via* the suppression of NLRP3 inflammasome activation.

In this study, we employed RSV to infect immunocompromised adult mice, thereby inducing airway injury. Subsequently, Calycosin was analyzed for its role in ameliorating RSV-infected mice and its potential underlying mechanisms were investigated. This study aims to provide foundational data supporting the application of Calycosin in the antiviral domain.

Materials and Methods

Animals

The 8-week-old female BALB/c mice (weights, 18–20 g) were procured in SPF (Beijing) Biotechnology Co., Ltd. After 1 week of adaptive feeding under SPF conditions, the mice were rendered immunocompromised through *intraperitoneally* injecting cyclophosphamide (CYP) at 100 mg/kg (Zhou

et al. 2024). The Animal Welfare and Ethics Committee of Wuhan Bestcell Model Biotechnology Co., Ltd approved the present work (BSMS. 2025-05-09A).

Grouping and treatment

Mice were randomized as the control, Calycosin, RSV, RSV+Calycosin, RSV+Nigericin and RSV+Calycosin+Nigericin groups. The RSV Long strain was acquired from Viruses Institute of Chinese Academy of Preventive Medicine. On the fifth day following CYP injection, animals received administration of 2% sodium pentobarbital (40 mg/kg) for anesthesia and were subsequently infected *via* intranasal instillation with 100 μ l of RSV (titer: 1.0×10^8 PFU/ml) (Zhou et al. 2024). Control mice were given normal saline at the same amount *via* the same method. Calycosin (dissolved in 0.5% dimethyl sulfoxide; Aladdin, Shanghai, China) was administered *via* intraperitoneal injection at a dose of 40 mg/kg once daily for 5 days (Song et al. 2017), starting 2 h after RSV infection. Nigericin (MedChemExpress, Monmouth, USA), an NLRP3 agonist, was administered intraperitoneally at a dose of 5 mg/kg once every other day, 30 min prior to Calycosin administration (Yang et al. 2021; Huang D et al. 2022). The control groups received an equivalent volume of either 0.5% dimethyl sulfoxide or normal saline.

Measurement of airway hyperresponsiveness (AHR)

AHR 24 h after the final Calycosin injection, airway resistance was measured using Whole-body plethysmography system (WBP-8M; TOW-INT Tech, Shanghai, China). Initially, mice were exposed to aerosolized normal saline in an aerosol chamber, followed by exposure to varying concentrations (3.125–50 mg/ml) of Methacholine (Aladdin, Shanghai, China) solution to stimulate airway. Each concentration was stimulated for 3 min followed by 2 min of rest, and the enhanced pause (Penh) value during these 5 min was recorded.

Inflammatory cells count

Mice were euthanized *via* overanesthesia induced by sodium pentobarbital (2%, 130 mg/kg), followed by the collection of lung tissues. After tracheal intubation, the right lung tissue was lavaged with precooled (4°C) PBS, and the bronchoalveolar lavage fluid (BALF) was subsequently collected. BALF samples underwent centrifugation (160 g, 10 min, 4°C) to collect supernatants, which were preserved under -80°C , while PBS was added to resuspend cell pellet. Afterwards, cell suspension aliquots (10 μ l) were used for total cell counting, and another 20 μ l was applied to slides for Wright- Giemsa staining (Beyotime, Shanghai, China). Subsequently, macrophages, lymphocytes, neutrophils, and eosinophils were counted.

Enzyme-linked immunosorbent assay (ELISA)

BALF supernatants collected above were utilized to quantify interferon (IFN)- γ , interleukin (IL)-1 β , IL-6, and IL-18 levels in BALF via ELISA, as recommended (Nanjing Jiancheng, Nanjing, China). Part of left lung was minced, homogenized, and centrifuged to obtain supernatants. Later, ELISA was conducted to determine IL-1 β and IL-18 contents within lung tissue.

Histopathological analysis

Mouse left lung tissues underwent 24 h of fixation within 4% paraformaldehyde before dehydration and paraffin embedding. Paraffin-embedded tissue blocks were sectioned in 5 μ m thick slices, followed by hematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS) staining following specific protocols (Servicebio, Wuhan, China). Following dehydration, neutral gum was added to mount these sections, and a microscope was then employed to evaluate pathological changes of the mouse lung tissues. The tissue inflammatory response was assessed using a double-blind scoring method as follows: Score 0, no inflammatory cell exudation; Score 1, mild inflammatory cell exudation; Score 2, exudative cells forming a monolayer cell ring; Score 3, exudative cells forming 2–4 layers of cell rings; Score 4, exudative cells forming more than 4 layers of cell rings (Zang et al. 2011). PAS staining was evaluated using the following scoring system: Score 0, no goblet cell; Score 1, fewer than 25% goblet cells; Score 2, 25–50% goblet cells; Score 3, 50–75% goblet cells; Score 4, more than 75% goblet cells (Lu et al. 2016). At least five distinct visual fields were selected from each section for evaluation.

Immunohistochemical staining

The deparaffinized paraffin-embedded sections underwent antigen retrieval and endogenous peroxidase inhibition. After 30 min of blocking using 3% bovine serum albumin under ambient temperature, sections received overnight primary antibody incubation against mouse NLRP3 (1:200; Beyotime, Shanghai, China) under 4°C, and then additional 50 min of respective secondary antibody incubation (1:50; Beyotime, Shanghai, China) under ambient temperature. Color development was performed by applying a DAB chromogen solution, before 3 min of hematoxylin counterstaining. After dehydration and mounting, the expression and distribution of NLRP3 within mouse lung tissue were analyzed with the light microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNAiso Plus reagent (Takara, Dalian, China) was adopted for isolating total RNA in mouse lung tissue. Subsequently,

total RNA was prepared into first-strand cDNA with PrimeScript RT Reagent Kit (Takara, Dalian, China) through reverse-transcription. Later, SYBR Green Master Mix (Applied Biosystems, Foster City, USA) was employed for PCR analysis, with synthesized cDNA as the template. The program involved 10 min of initial denaturation under 95°C; 15 s under 95°C and 1 min under 60°C for 40 cycles. qRT-PCR primers used for the analysis are as follows: RSV nucleoprotein (RSV-N) forward (5'-3') AGATCAACTTCTGTCATCCAGCAA and reverse (5'-3') TTCTGCACATCATAATTAGGAGTATCAAT; GAPDH forward (5'-3') CGTGTTC TACCCCAATGT and reverse (5'-3') TGT-CATACTTGGCAGGTTTCT. The aforementioned primers were synthesized by Sangon Bioengineering (Shanghai) Co., Ltd. The relative expression level of RSV-N in mouse lung tissue was determined by $2^{-\Delta\Delta C_t}$ approach.

Western blotting analysis

Protein was extracted from mouse lung tissue using RIPA buffer, and protein concentration was determined by BCA method. Proteins were separated by gel electrophoresis, transferred to the membrane, and blocked for 2 h at room temperature. The membranes were then incubated with anti-mouse NLRP3 antibody (1:500; Beyotime, Shanghai, China), Cleaved-caspase-1 antibody, cleaved N-terminal Gasdermin D (GSDMD-N) antibody, IL-1 β antibody, and GAPDH antibody (1:1000; Cell Signaling Technology, Danvers, USA) overnight at 4°C. After washing, membranes underwent additional 1 h of secondary antibody incubation under ambient temperature. Finally, chemiluminescence detection was carried out for protein band visualization in a darkroom, whereas band grayscale intensity was analyzed with image analysis software.

Statistical analysis

All experiments were carried out with five biological replicates. Statistical analyses were completed with GraphPad Prism 8 software. Results were represented by mean $\pm$ standard deviation (SD). An unpaired *t*-test was employed for the comparison between two independent groups, while ANOVA was utilized for comparisons involving multiple groups. *p* < 0.05 suggested significant differences.

Results

Calycosin suppressed RSV replication and ameliorated lung inflammation of RSV-infected mice

We successfully established the adult BALB/c mouse model of RSV infection following the suppression of immune

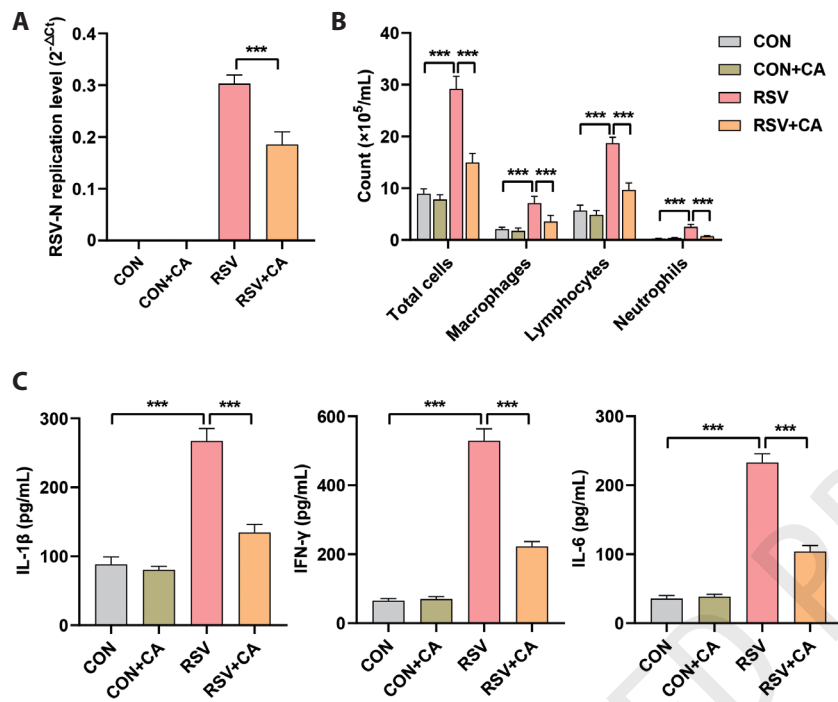


Figure 1. Calycosin suppressed RSV replication and alleviated inflammatory responses in mice lung tissues. A RSV infection model was established by inducing immunodeficiency in mice using cyclophosphamide and infecting them with RSV through intranasal instillation. The mice were continuously treated with 40 mg/kg/day Calycosin for 5 days. **A.** qRT-PCR was employed to quantify RSV-N replication levels in the lung tissues of mice. **B.** The total cell count, as well as the specific counts of macrophages, lymphocytes, and neutrophils, was determined in the BALF of mice. **C.** ELISA was utilized to assess the concentrations of IL-1 β , IFN- γ , and IL-6 in BALF of mice. CON, Control. CA, Calycosin. $n = 5$. *** $p < 0.001$.

function *via intraperitoneal* injection of CYP. The RSV-N was detected in lung tissue of RSV-infected mice, yet it was scarcely detectable in uninfected mice. Significantly, the intervention of Calycosin markedly inhibited RSV replication

in the lung tissues of RSV-infected mice (Fig. 1A). Additionally, total cell count, together with macrophage, lymphocyte, and neutrophil quantities within BALF, evidently elevated among RSV-infected mice (Fig. 1B). Additionally, IFN- γ , IL-1 β , and IL-6 contents inside BALF significantly increased

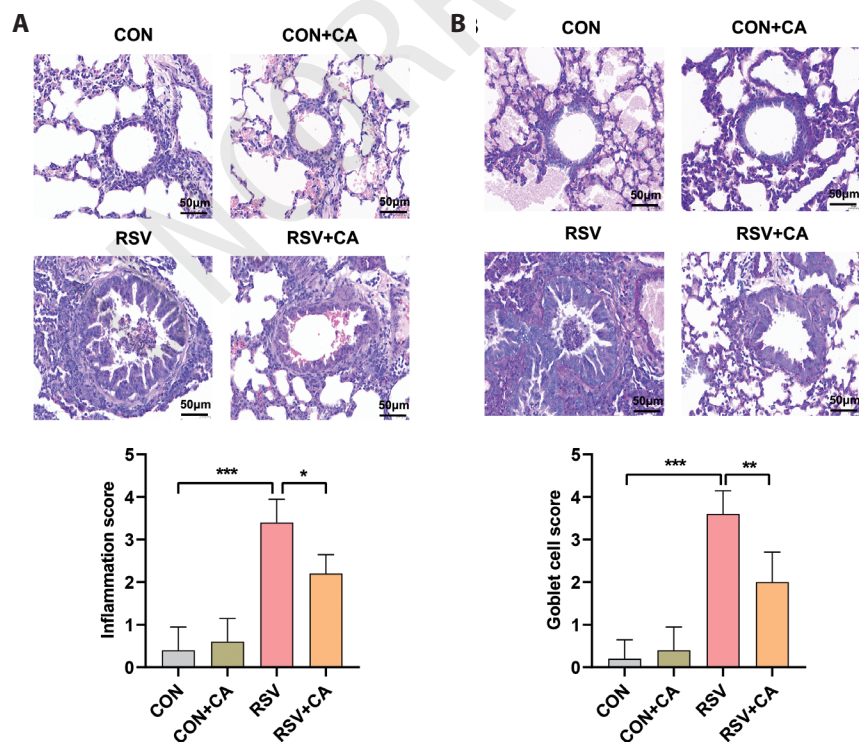


Figure 2. Calycosin alleviated airway injury in RSV-infected mice. **A.** HE staining was employed to evaluate pathological damage in the lung tissue of mice. Scale bar = 50 μm . **B.** PAS staining was utilized to assess goblet cell hyperplasia and mucus secretion in murine lung tissue. Scale bar = 50 μm . **C.** The whole-body plethysmography was used to monitor the Penh values in mice to evaluate airway resistance. CON, Control. CA, Calycosin. $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(Fig. 1C). Treatment with Calycosin effectively attenuated lung inflammation of RSV-infected mice, significantly reducing both inflammatory cell quantity and pro-inflammatory factor contents within BALF, without exerting any noticeable effect on normal mice (Fig. 1 B,C).

Calycosin ameliorated RSV-induced lung injury and airway resistance in mice

Subsequently, Calycosin was analyzed for its roles in airway injury of RSV-infected mice. We observed the pathological alterations within lungs in RSV-infected mice. Specifically, we found that the bronchial walls were thickened, alveolar spaces were markedly narrowed, inflammatory cell infiltration was present in the lung interstitium, and there was an increase in goblet cells along with mucus secretion (Fig. 2 A,B). Following Calycosin administration, the pathological damage to lung tissue in RSV-infected mice was mitigated, and the infiltration

of inflammatory cells and goblet cells were decreased. Furthermore, we observed that airway resistance in RSV-infected mice increased significantly with rising acetylcholine concentrations, whereas Calycosin treatment effectively attenuated this increase in airway resistance, although it had no effect on the airway resistance of uninfected control mice (Fig. 2C). These findings indicate that Calycosin treatment can significantly alleviate airway injury in mice infected with RSV.

Calycosin inhibited NLRP3 inflammasome activation of RSV-infected mice

Many RNA viruses, including RSV, and their components can activate NLRP3 inflammasome, thereby modulating inflammatory response (Choudhury et al. 2021). According to our observations, NLRP3 inflammasome was activated inside the lungs of RSV-infected mice, which was observed from the higher NLRP3, Cleaved-caspase-1, GSDMD-N, and

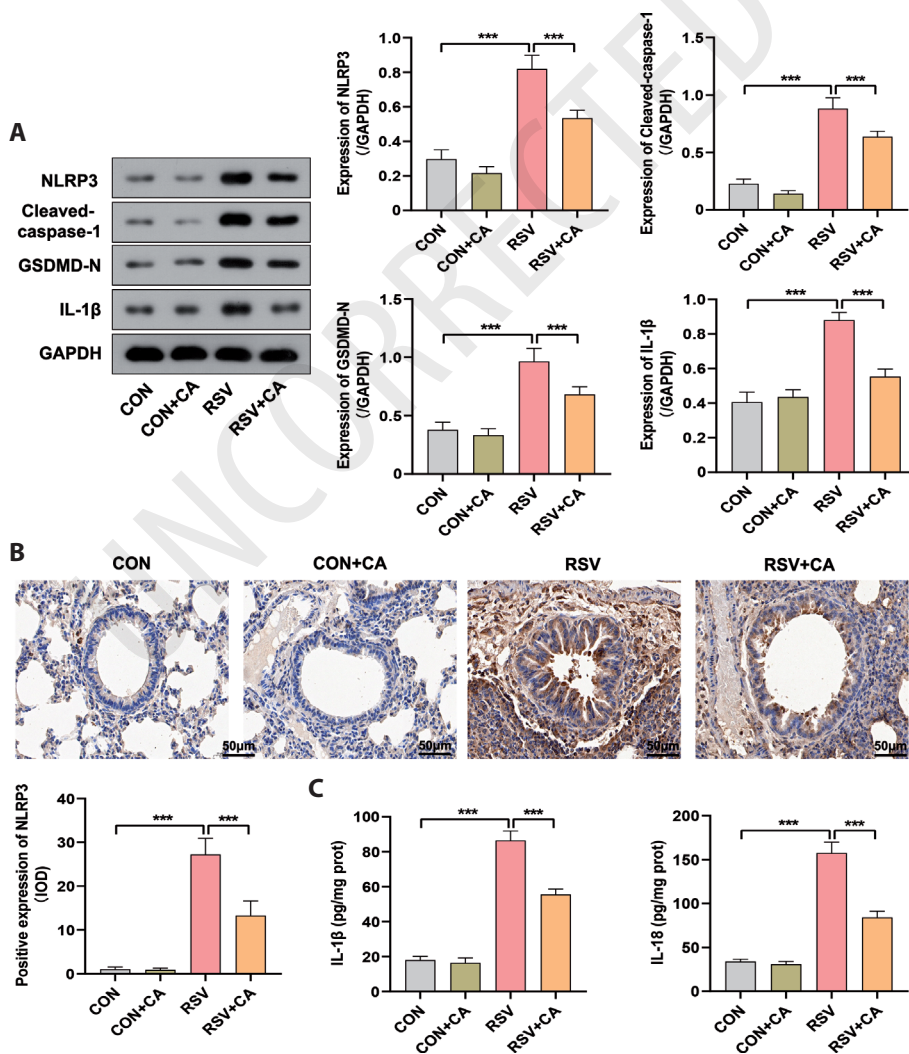


Figure 3. Calycosin suppressed NLRP3 inflammasome activation in lung tissues of RSV-infected mice. **A.** Western blotting analysis was employed to assess the protein expression levels of NLRP3, Cleaved-caspase-1, GSDMD-N, and IL-1β in the lung tissue of mice. **B.** Immunohistochemical staining was utilized to examine the distribution and expression of NLRP3 protein in the lung tissue of mice. **C.** ELISA was performed to quantify the concentrations of IL-1β and IL-18 in the lung tissue of mice. Scale bar = 50 μm. CON, Control. CA, Calycosin. *n* = 5. *** *p* < 0.001.

IL-1 β protein levels (Fig. 3A,B), and higher IL-1 β and IL-18 secretion (Fig. 3C). Following treatment with Calycosin, NLRP3, Cleaved-caspase-1, GSDMD-N, and IL-1 β levels, along with IL-1 β and IL-18 concentrations significantly decreased within lungs in RSV-infected mice (Fig. 3A-C).

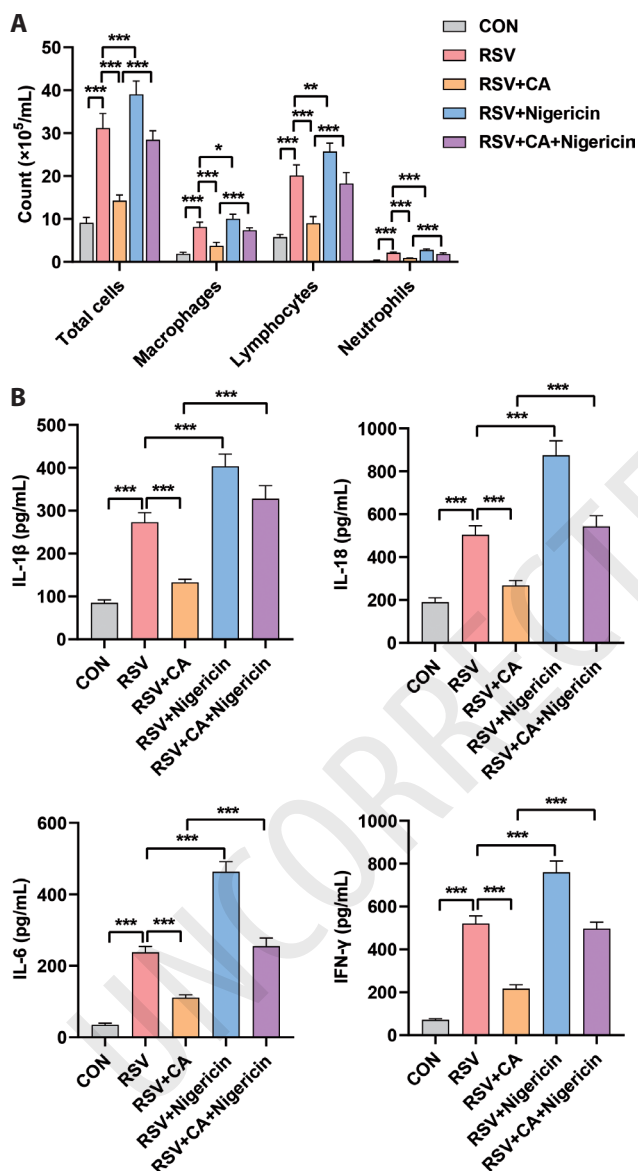


Figure 4. Nigericin attenuated the anti-inflammatory effect of Calycosin in the lungs of RSV-infected mice. RSV-infected mice were subjected to continuous intraperitoneal injection of 40 mg/kg/d calycosin for 5 days, or intraperitoneal injection of 5 mg/kg NLRP3 agonist nigericin 30 min prior to calycosin treatment (every other day). **A.** The total cell count, as well as the numbers of macrophages, lymphocytes, and neutrophils in the BALF of mice, were statistically analyzed. **B.** ELISA was performed to quantify the concentrations of IL-1 β , IFN- γ , IL-6, and IL-18 in the BALF of mice. CON, Control. CA, Calycosin. $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Therefore, we hypothesized that Calycosin may attenuate RSV infection-induced mouse airway inflammation by suppressing NLRP3 inflammasome activation.

NLRP3 inflammasome activation reversed Calycosin's effect on airway inflammation of RSV-infected mice

For validating our hypothesis, RSV-infected mice were treated with Calycosin and the NLRP3 agonist Nigericin. The results demonstrated that treatment with Nigericin alone in RSV-infected mice remarkably elevated total cell count, macrophage, lymphocyte, and neutrophil quantities in BALF (Fig. 4A). Additionally, IL-1 β , IL-18, IL-6, and IFN- γ contents markedly elevated (Fig. 4B). More importantly, co-intervention with Nigericin partially attenuated the inhibitory effect of Calycosin on the inflammatory response in lung tissues of RSV-infected mice. Subsequently, we further investigated lung tissue injury in mice. The findings revealed that RSV-infected mice exhibited more pronounced pulmonary pathological damage, a higher score of inflammatory cell infiltration (Fig. 5A), and elevated airway resistance following Nigericin intervention (Fig. 5B). Moreover, the protein expression levels of NLRP3, Cleaved-caspase-1, GSDMD-N, and IL-1 β in the lung tissue of mice were significantly upregulated (Fig. 5C). It is noteworthy that under the intervention of Nigericin, the efficacy of Calycosin in alleviating lung tissue inflammatory damage and airway resistance in RSV-infected mice was inhibited, and the inhibitory effect on NLRP3 inflammasome activation was partially reversed.

Discussion

This study represents the first investigation to reveal the improvement effect of Calycosin on airway inflammatory injury in RSV-infected mice and to elucidate the molecular mechanism by which Calycosin mitigates inflammatory response through the inhibition of NLRP3 inflammasome activation.

Traditional Chinese medicine and its natural products have demonstrated remarkable potential in anti-RSV therapy, characterized by unique advantages such as multi-target synergy, immune modulation, and minimal side effects. Basic research has indicated that Jerusalem artichoke (*Helianthus tuberosus* L.) polysaccharide not only suppresses the activity of RSV *in vitro* but also significantly inhibits RSV proliferation within lungs in RSV-infected mice, thereby alleviating lung tissue damage in mice (Wan et al. 2022). According to Qin et al. Baicalin suppressed NS1 and NS2 (RSV nonstructural proteins) transcription, as well as the translation of matrix protein RNA, consequently eliciting an innate immune response for inhibiting RSV replication (Qin et al. 2022). Calycosin-7-O- β -D-glucoside is a predominant

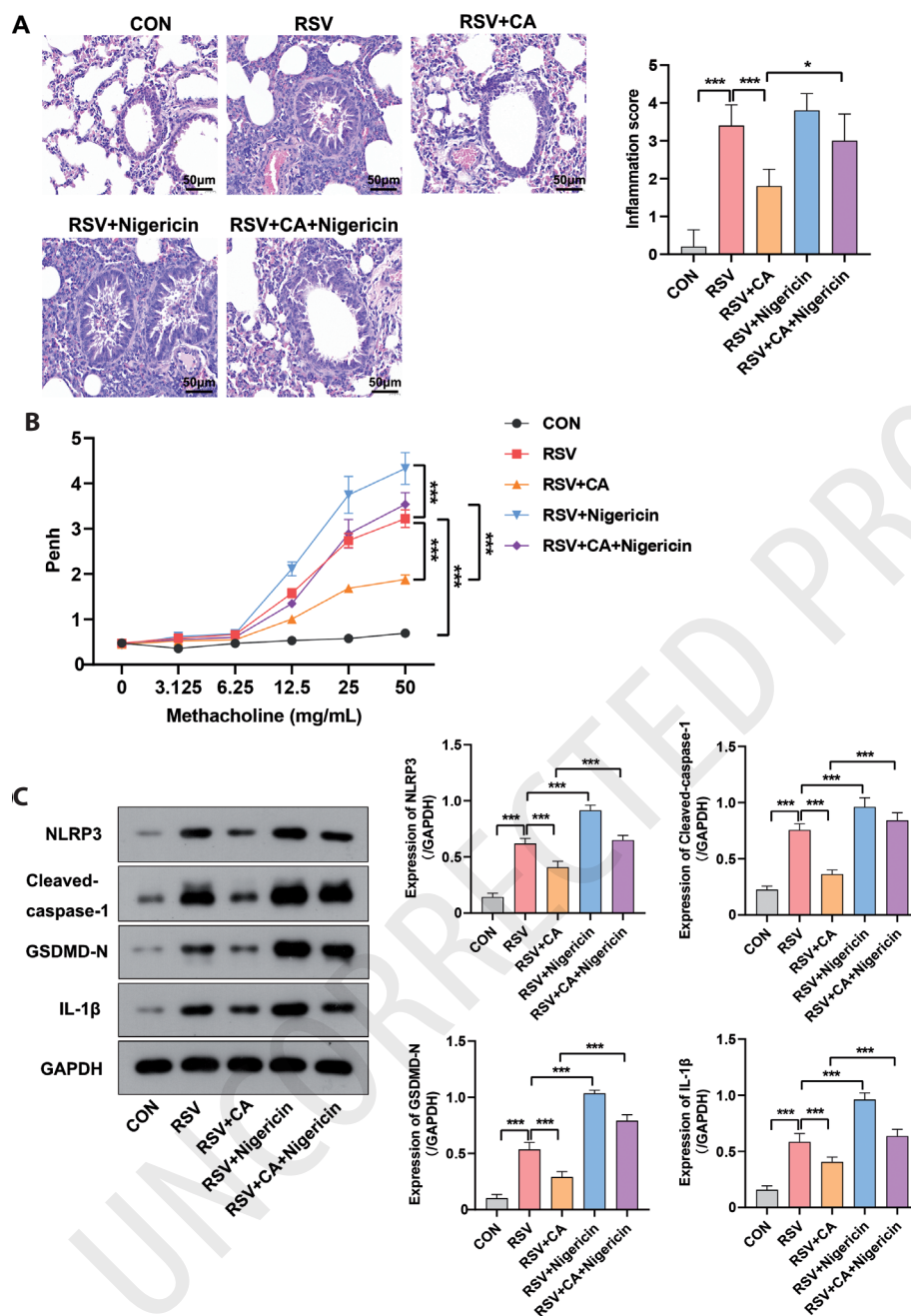


Figure 5. Nigericin reversed the protective effect of Calycosin against lung injury in RSV-infected mice through activation of the NLRP3 inflammasome. **A.** HE staining was employed to evaluate pathological damage in the lung tissue of mice. Scale bar = 50 μm. **B.** The whole-body plethysmography was used to monitor airway resistance of mice. **C.** Western blotting analysis was employed to determine the protein expression levels of NLRP3, Cleaved-caspase-1, GSDMD-N, and IL-1β in the lung tissue of mice. CON, Control. CA, Calycosin. $n = 5$. * $p < 0.05$, *** $p < 0.001$.

flavonoid storage form in *Astragalus membranaceus*. It has a basic structure that Calycosin possesses a glucose group attached to the 7th hydroxyl group of Calycosin. Research has demonstrated that calycosin-7-O-β-D-glucoside exhibits antiviral properties, effectively reducing the coxsackievirus B3 viral load in the hearts of mice and alleviating virus-induced myocarditis (Zhu et al. 2009). It has been demonstrated that calycosin-7-O-β-D-glucoside undergoes *in vivo* metabolism primarily through pathways such as deglycation, dehydroxylation, and demethylation. Notably, its metabolite Calycosin

exhibits stronger antiviral activity compared to the parent compound (Chen L et al. 2011). Furthermore, Tian et al. confirmed that Calycosin achieves a higher tissue distribution level than calycosin-7-O-β-D-glucoside in organs including the liver, kidney, and heart (Tian X et al. 2018). Although calycosin exhibits significant antiviral activity against both coxsackievirus B3 and human immunodeficiency virus, its effects on RSV have not been well defined. This study represents the first report demonstrating that Calycosin inhibits RSV replication in mouse lung tissue.

Following RSV infection, patients typically present with prominent upper and lower respiratory tract symptoms as well as various complications (Duan et al. 2024). Given that mice can develop lower respiratory tract lesions analogous to those observed in humans, mouse models of RSV infection are frequently employed to investigate the molecular and cellular mechanisms underlying RSV pathogenesis (Shilovskiy et al. 2021). RSV-infected mice exhibit severe lung inflammation characterized by increased infiltration of macrophages, lymphocytes, and neutrophils, excessive mucus secretion in the lungs, and robust production of proinflammatory cytokines (Makino et al. 2021; Zhou et al. 2024). In this study, immunocompromised BALB/c mice infected with RSV exhibited severe pneumonia and increased airway resistance. However, Calycosin treatment significantly mitigated airway inflammatory injury in RSV-infected mice. These results unequivocally demonstrate that Calycosin holds significant potential for anti-RSV applications; however, its mechanism of action requires further elucidation.

Upon entering the host, RSV is recognized by pattern recognition receptors, which promote the expression of NLRP3 (Huang Y et al. 2024). Furthermore, RSV induces NLRP3 oligomerization *via* pathways such as ion flux disorder and mitochondrial damage. This process facilitates the recruitment of ASC to bind with NLRP3, as a result, the inflammasome complex together with Caspase-1 is formed (Segovia et al. 2012). Following this, Caspase-1 experiences self-cleavage for generating Cleaved-caspase-1, which is responsible for processing pro-IL-1 β and pro-IL-18 in corresponding mature forms and cleaving GSDMD to release its N-terminal domain. GSDMD-N translocates to cell membrane, where it forms pores, causing cell swelling, lysis, IL-1 β and IL-18 secretion, and inflammatory response amplification (Gros Lambert and Py 2018; Sayaf et al. 2024). Excessive or sustained activation of the NLRP3 inflammasome may result in immunopathological damage to tissues. Research has demonstrated that RSV infection triggers overactivation of the NLRP3 inflammasome in normal human lung epithelial cells. Furthermore, the suppression of NLRP3 inflammasome overexpression has been shown to effectively mitigate pneumonia in RSV-infected mice (Cheng et al. 2023). Study has demonstrated that Calycosin can mitigate sepsis-induced acute lung injury in young rats by suppressing the inflammatory response mediated through the activation of the NLRP3 inflammasome (Chen G et al. 2021). Likewise, Xia et al. (2021) reported that Calycosin exhibited a substantial therapeutic effect on lung inflammatory injury, with the underlying mechanism being associated with the inhibition of mitochondrial ROS-mediated activation of the NLRP3 inflammasome. These findings collectively indicate that Calycosin plays a crucial role in modulating the activation of the NLRP3 inflammasome. From our results, Calycosin treatment markedly suppressed NLRP3

inflammasome activation within lungs in RSV-infected mice. However, co-treatment with NLRP3 agonist Nigericin abolished Calycosin's functions in lung inflammatory injury and airway resistance in mice. Consequently, Calycosin mitigates RSV infection-induced airway injury through inhibition of the inflammatory response mediated by NLRP3 inflammasome activation.

In conclusion, Calycosin exhibits a significant therapeutic effect on airway inflammatory injury induced by RSV infection, and its mechanism of action is associated with the suppression of inflammatory responses mediated by NLRP3 inflammasome activation. Nevertheless, given the limited number of studies investigating the anti-RSV effects of Calycosin, additional research data are required to substantiate the conclusions drawn in this study. In this study, CYP was employed to suppress the immune function of adult mice, thereby enhancing their susceptibility to RSV. Nevertheless, this model is unable to fully replicate the immune status of RSV infection in infants and young children. Consequently, in future research, the efficacy of Calycosin requires further verification in young animal models with immature immune systems to comprehensively assess its application prospects in diverse host states. This study employed solely a single dose of Calycosin to investigate its therapeutic efficacy against RSV. Such a methodology has limitations as it fails to elucidate the dose-effect relationship of Calycosin in RSV infection. In subsequent studies, leveraging the findings of this study, we will design multi-dose gradient experiments of Calycosin to determine the optimal dose window and the dose-effect relationship of Calycosin in the treatment of RSV infection.

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Conflict of interest. The authors declare no competing interests.

Ethics approval. The Animal Welfare and Ethics Committee of Wuhan Bestcell Model Biotechnology Co., Ltd approved the present work (BSMS. 2025-05-09A).

Author contributions. XW, HJ designed the research study. Material preparation, data collection and analysis were performed by XW, SW and HJ. The first draft of the manuscript was written by XW and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability. Data are available from the corresponding author upon reasonable request.

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