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## Original Research Article

# Bryostatin 1 alleviates respiratory syncytial virus pneumonia in a mice model via down-regulation of inflammatory cytokines

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## Abstract

***Purpose:** To investigate the effect of bryostatin 1 on respiratory syncytial virus (RSV) infection in vitro in lung alveolar cells and in vivo in a mice model.*
***Methods:** RSV infection in the mice was induced by the administration of 2 x 106 PFU viral particles intranasally in the left nostril. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting were used for the determination of changes in interleukin expression.*
***Results:** Bryostatin 1 treatment of RSV-infected BEAS-2B cells significantly ($p < 0.05$) inhibited viability. The mortality of mice infected with RSV markedly decreased on treatment with bryostatin 1. The pulmonary damage induced by RSV infection was also prevented in mice treated with bryostatin 1. Treatment of the RSV infected mice with bryostatin 1 caused a dose-based suppression of IL 1β/ 18 and TNF α generation ($p < 0.05$). Bryostatin 1 pre-treatment at doses 2, 6 and 12 mg/kg led to reduction of NLRP3, ASC and caspase 1 proteins, as well as a significant decrease in the expression of mRNA corresponding to NLRP3, ASC and caspase 1 ($p< 0.05$).*
***Conclusion:** The results demonstrate that bryostatin 1 treatment of RSV-infected BEAS-2B cells prevents reduction in its viability. Moreover, pre-treatment of RSV-infected mice with bryostatin 1 improves mortality and prevents pulmonary tissue damage by down-regulating NLRP3 activation. Therefore, bryostatin 1 may be an option for the development of an effective treatment for pneumonia.*

***Keywords:** Pneumonia, Pharyngitis, Inflammasome, Inflammatory cytokine*

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## INTRODUCTION

Respiratory syncytial virus (RSV) contains negative-stranded RNA as the genetic material, and belongs to the family of *Paramyxoviridae*. The virus is responsible for severe respiratory tract infections in people of different age groups particularly in children [1]. The host-virus interaction produces reactive oxygen species which act as the mediators of epithelial cell and pulmonary tissue damage following RSV infection [2]. Respiratory syncytial virus infection causes high production of ROS which subsequently induces secretion of various interleukins [3]. Increased generation of inflammatory cytokines by RSV leads to

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imbalance between cytokine types 1 and 2 which are associated with pathogenesis of pneumonia [4].

The immune pathways are involved in the development of various diseases like diabetes type 2 [5], atherosclerosis [6] and Alzheimer's disease [7]. Inflammasome consists of gene containing nucleotide-binding domain and leucine-rich repeat (NLR) domain consisting of pyrin-3 protein (NLRP3), an adaptor protein with speck-like C-terminal caspase (ASC) and effector protein caspase-1 [8]. Activation of NLRP3 inflammasome is associated with various types of stimuli like viral and bacterial infections [9,10]. It is reported that *S. aureus* catalyses production of IL-1β and activates caspase-1 through the activation of NLRP3 [11]. The NLRP3 activation plays significant role in the development of pneumonia in *S. aureus*-infected murine model [12]. These findings suggest that NLRP3 inflammasome can act as an important target for the treatment of pneumonia.

Chemical investigation of the marine invertebrate *Bugula neritina* belonging to the phylum Ectoprocta afforded bryostatin 1 [13]. Biological screening of bryostatin 1 revealed its promising immune system development property and haemopoisis stimulation potential [13]. Bryostatin 1 inhibits the growth and proliferation of tumor cells induced by phorbol ester [14]. In cultures of ovary carcinoma cells exposure to bryostatin 1 reduced proliferation through apoptosis induction [13]. Moreover, growth and proliferation of various types of cancer cells like melanoma, leukaemia, kidney and pulmonary cells is inhibited on exposure to bryostatin 1 [15]. The study involving mice has demonstrated direct relationship of *in vitro* anti-tumor activity of bryostatin 1 with the *in vivo* results [15]. In the present study, the effect of bryostatin 1 on RSV induced pneumonia in the mice model was investigated *in vivo*.

## EXPERIMENTAL

### Cell culture and culture

The BEAS-2B, bronchio-epithelial cell line was supplied by the American Type Culture Collection (ATCC; Rockville, MD, USA). The cell culture was performed in DMEM medium containing fetal bovine serum (12%) and L-glutamine (2 mM). The antibiotics, penicillin (100 U/mL) and streptomycin (100 mg/ml) were also added into the medium. The RSV-A2 viral strains were grown in HEp-2 cells [10]. For *in vitro* studies RSV was used in different concentrations (multiplicity of infection, MOI= 4.5-0.01), and immunoplaque assay was used for determination of RSV titers.

### Cell viability assay

RSV induced changes inBEAS-2B cell viability was analysed by 3(4,5dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, about 200 µL of the cell suspension containing cells at 2 x $10^5$/Ml were put into 96-well culture plates. After initial culture for 24 h, the cells were incubated with RSV-A2 and then exposed to 2, 4, 6, 8 10 and 12 µM of bryostatin 1 for 72 h. Then, 30 µL of MTT solution (5 mg/mL) was put into the wells and cells were subjected to 4 h of incubation at 37˚C. Dimethyl sulfoxide (150 µL) was added to well for dissolving formazen crystals. The microtitre plates were shaken for 10 min in shaker and then absorbance was recorded by Bio-Rad iMark plate reader.

### Animals and establishment of pneumonia model

The fifty female BALB/c mice (8-10 week old) were obtained from the Nanjing University Animal Research Center (Nanjing, China). The mice were kept in sterilized cages under controlled conditions of 24 ± 2˚C temperature and 60% humidity. The mice were exposed to 12h light and dark cycles and were given free access to rodent chow and water. Approval for the animal study was obtained from the Animal Ethics Committee, Tianjin Medical University (TMU/009/17/AS). The experimental procedures were carried out according to guidelines issued by the Laboratory Animal Care and Use Committee, National Institute of Health, China [10].

The mice were divided into five groups of 10-each for the establishment of pneumonia mice model. Four groups of mice were subjected to isoflurane anaesthesia and then intranasally infected with RSV at 5 x $10^6$ PFU in 60 µL of PBS. The mice in normal control (sham) group were administered with PBS alone. Bryostatin 1 was injected to the mice in three treatment groups (2, 6 and 12 mg/kg) intraperitoneally 12 h before the RSV infection. The mortality in mice of various groups was recorded over 72 h after RSV infection.

### Histopathological examination

Five mice from each of the group were sacrificed at 72 h of RSV infection under pentobarbital anaesthesia. The lungs, bronchoalveolar lavage

fluid and blood samples of the mice were then collected. The lungs were washed two times with 250 μL of ice-cold PBS using cannula through tracheal tubes to collect the aliquots. The pulmonary tissues were then subjected to fixing with 8% formalin, subsequently paraffin embedded and then sliced into thin sections of 2 μm thickness. The sections were subjected to blocking with 4% bovine serum albumin and then stained with hematoxylin and eosin (H&E) staining. Light microscope (CKX31; Olympus, Tokyo, Japan) was used for the examination of stained tissues.

**Western blot analysis**

The pulmonary tissues of mice were lysed using lysis buffer [tris-hydrochloride (10 mM), EDTA (1.1 mM) and sucrose (260 mM), pH 7.6, mixed with aprotinin (16 μg/mL), leupeptin (6 μg/mL), $PhCH_2SO_2F$ (0.2 mM), sodium fluoride (1.1 mM)and sodium vanadate (1.1 mM)]. The lysates were centrifuged for 25 min at 12000 x g to collect the supernatant. The protein concentration in the supernatant was determined using bicinchoninic acid assay (BCA). The protein (20 μg) samples were resolved by electrophoresis on SDS-PAGE (12 %) and subsequently transferred to the polyvinylidene fluoride membranes.

The membranes were subjected to incubation overnight at 4 ˚C with primary antibodies followed by PBS washing. Then membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies at room temperature for 1 h. Finally, blots were visualized by chemiluminescence and autoradiography. LabWorks 4.5 analysis software (UVP, Inc., Upland, CA, USA) was used to perform quantification of bands in the western blots. The primary antibodies used were: anti-NLRP3, anti-ASC, anti-Caspase-1, anti-pro-Caspase-1, IL-1β, IL-18, TNF-α and anti-GAPDH (all obtained from Abcam, Cambridge, MA, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

The collection of bronchoalveolar lavage fluid from the mice was carried out by instilling 250μLof PBS two times intratracheally. The pulmonary tissues were excised and then subjected to homogenization with PBS. The homogenised tissues were subjected to 15 min of centrifugation at 4˚C at 2000 x g to obtain the supernatant. The IL-1β, IL-18 and TNF-α cytokine level in the supernatants was measured using ELISA kits.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

The pulmonary tissues were ground using liquid nitrogen and mixed with 1 ml TRIzol (10606ES60; Yeasen, Shanghai, China) for lysis. The RNase-free DNase I (Takara Bio Inc.) was employed for synthesis of DNA from the RNA samples. Reverse transcription of 1 μgRNA samples to cDNA was carried out using 20 μL of mixture reaction mixture. The mixture contained 30 mM magnesium chloride (3 μL), AMV reverse transcriptase (15.5μL), 10xbuffer for revere transcription (2.2 μL), 8 mM dNTP mixture (2.2μL), inhibitor for RNase (0.6 μL), and Oligo (dT) primers (0.5 μg). The reactions were carried out using the following sequence: 75 min at 45 ˚C then 96 ˚C for 7 min. The 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) employing SYBR Premix Ex Taq (Takara Bio, Inc) was used for qPCR in accordance with the instructions of the manufacturer. The mixture for PCR reaction contained 2 μg cDNA as template, 10x buffer, ligase, 2 mmol/L magnesium ions, dNTP, 200 μmol/L of each dNTP, 18 pmol of each primer and nuclease-free water. The sequence for PCR involved 95 ˚C for 6 min, then 35 cycles for 40 s at 95 ˚C, for 55 s at 58 ˚C and 40 s at 70 ˚C.

**Statistical analysis**

The data are presented as mean ± SD (n = 3). Differences were determined statistically using Student's *t*-test and one way ANOVA, and were considered statistically significant *at* $p < 0.05$.

## RESULTS

**Inhibition of RSV replication by bryostatin 1**

Bryostatin1 exposure did not affect the viability of BEAS2B cells at 2 and 12 μM (Figure 1 A). There was no significant change in BEAS-2B cell viability during 72 h of incubation with 2and 12 μM of bryostatin 1. On the other hand, BEAS-2B cell viability was reduced significantly ($p$<0.05) on RSV infection from 0.25 to 4.5 MOI (Figure 1 A). Increase in RSV MOI from 0.25 to 4.5 MOI reduced viability of BEAS-2B cells from 92 to 29% when compared to 100% in control. Incubation of RSV infected BEAS-2B cells with bryostatin 1 inhibited reduction of viability in concentration-based manner (Figure 1 B). At 12 μM, bryostatin completely inhibited RSV induced reduction of BEAS-2B cell viability.

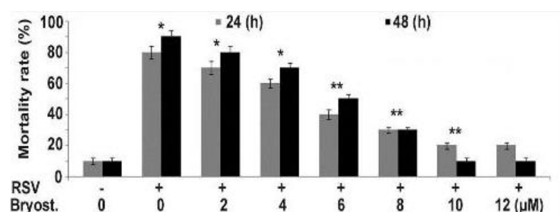


**Figure 1:** Bryostatin inhibits RSV induced reduction in cell viability. (A) The cells incubated with indicated doses of bryostatin 1 or RSV were analysed by MTT assay. (B) RSV induced reduction of cell viability inhibition by bryostatin; **p* < 0.05 and ***p* < 0.01 versus negative control cells

## Bryostatin 1 suppresses RSV-induced mortality in mice

*RSV* infection significantly ($p < 0.05$) increased mortality in mice in comparison to those in the control group (Figure 2). Treatment of the RSV infected mice with bryostatin 1 caused a marked decrease in RSV-induced increase in mortality rate. A dose-based improvement in RSV induced mortality was caused in mice on treatment with bryostatin1 for 72 h. At 12 mg/kg, bryostatin1 treatment completely inhibited RSV induced mortality in the mice.

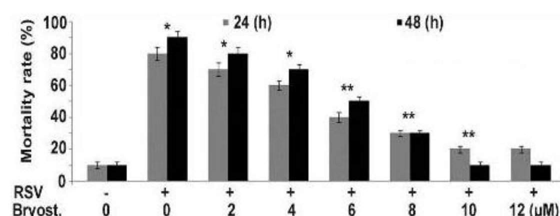


**Figure 2:** Bryostatin 1 improves mortality rate in RSV infected mice. Pre-treatment of the RSV infected mice with bryostatin 1suppresses RSV induced mortality; *$p$<0.05 and **$p$<0.01 versus control group

## Bryostatin protects RSV induced lung tissue damage in mice

Histopathological analysis showed that RSV infection caused a marked damage in pulmonary tissues of mice (Figure 3). In the RSV infected mice pulmonary tissues showed aggregation of inflammatory cells, breakdown of alveoli and degraded tissues. Bryostatin pre-treatment of the RSV infected mice prevented damage to pulmonary tissues markedly in comparison to the untreated group. The prevention of RSV induced lung tissue damage by bryostatin was dose dependent with maximum effect at 12 mg/kg.

## Suppression of RSV induced inflammatory cytokine secretion by bryostatin

In the RSV infected mice generation of IL-1β, IL-18 and TNF-α was markedly higher in the pulmonary tissues as well as in BALF than those in the normal mice (Figure 4). Treatment of the RSV infected mice with bryostatin1 caused dose-based suppressions of IL-1β, IL-18 and TNF-α generation in pulmonary tissues as well as BALF. The RSV infected mice, pre-treated with 2, 6 and 12 mg/kg of bryostatin showed significant suppression of IL-1β, IL-18 and TNF-α level. The generation of IL-1β, IL-18 and TNF-α in RSV infected mice was suppressed very close to the control group on treatment with 12 mg/kg of bryostatin1.

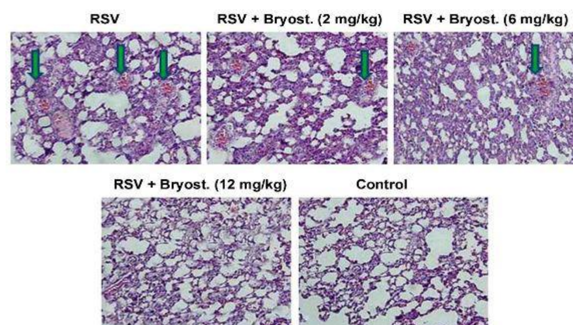


**Figure 3:** Effect of bryostatin on RSV induced tissues damage in mice. The RSV infected mice were pre-treated with bryostatin at doses 2, 6 and 12 mg/kg. Histopathology of pulmonary tissues was assessed using H&E (hematoxylin and eosin) staining. (Magnification, x250)

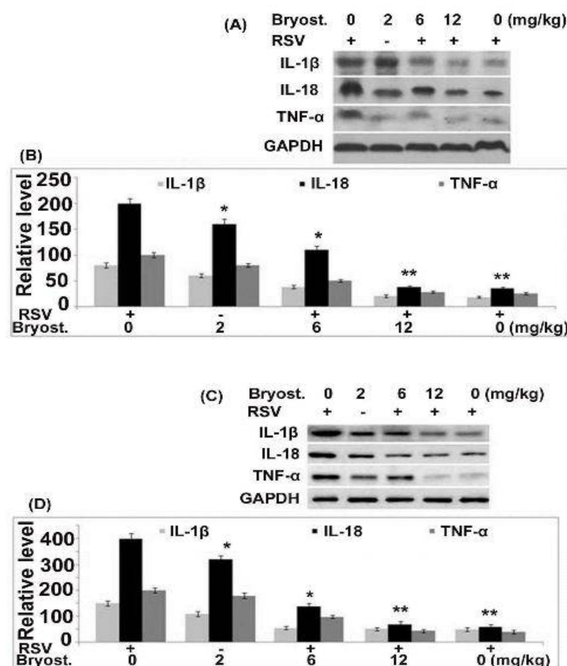


**Figure 4:** Bryostatin reduced RSV mediated generation of cytokines. In RSV infected mice generation of IL-1β, IL-18 and TNF-α (A,B) in pulmonary tissues and (C,D) BALF was measured by western blotting. *$p$<0.05 and **$p$<0.01 versus control group

**NLRP3 activation inhibition by bryostatinin RSV infected mice**

Western blotting revealed that bryostatin pre-treatment reduced the expression of proteins corresponding to NLRP3, ASC and caspase-1 in the RSV infected mice (Figure 5 A). Bryostatin pre-treatment of the RSV infected mice at 2, 6 and 12 mg/kg doses lead to reduction of NLRP3, ASC and caspase-1 proteins in dose based manner. The suppression of proteins was maximum in the RSV infected mice pre-treated with 12 mg/kg dose of bryostatin. In the RSV infected mice a markedly higher level of mRNA corresponding to NLRP3, ASC and caspase-1 was observed by RT-PCR (Figure 5 B). Pre-treatment of the mice with bryostatin caused a significant reduction in the expression of mRNA corresponding to NLRP3, ASC and caspase-1. The reduction of mRNA was almost comparable to those of control group at 12 mg/kg dose of bryostatin.

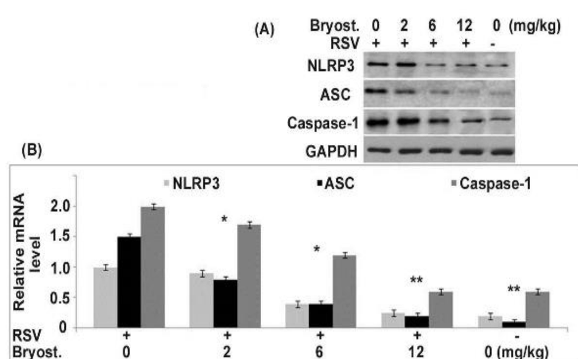


**Figure 5:** Bryostatin inhibits RSV induced NLRP3 activation. (A) Western blotting and (B) RT-PCR assays were used for determination of protein and mRNA expression in the RSV infected mice pretreated with bryostatin. *$P$<0.05 and **$p$<0.01 versus control group

## DISCUSSION

The present study demonstrated the effect of bryostatin1 on viability of RSV infected BEAS-2B cells *in vitro* and RSV induced pneumonia in the mice model. The study showed that bryostatin1 pre-treatment markedly reduced viability of RSV infected BEAS-2B cells and prevented RSV induced pneumonia in the mice model. Pre-treatment of RSV infected mice with bryostatin1 prevented pulmonary tissue damage through suppression of inflammatory cytokine generation and down-regulation of NLRP3 activation.

Natural products obtained from diverse sources like plants, microorganisms and animals have shown wide range of biological activities without causing any harmful effect. These compounds have shown significant result in the protection of *S. aureus* induced pneumonia in the mice models [16-18]. In the mice model of *S. aureus* induced pneumonia treatment with natural product has shown a significant decrease in mortality [19]. Bryostatin 1 is a natural product obtained from a marine, *Bugula neritina* of the phylum Ectoprocta [13]. In the present study, RSV infection significantly enhanced the viability of BEAS-2B cells *in vitro*. Exposure of RSV infected BEAS-2B cells to bryostatin 1 caused a concentration-based reduction of BEAS-2B cell viability. These findings suggest that bryostatin 1 exhibits inhibitory effect on RSV replication in BEAS-2B cells. In the mice model of RSV induced pneumonia, a marked increase in The RSV induced increase in mortality of mice was decreased significantly on treatment with bryostatin 1.

In the mice model of *S. aureus* pneumonia, therapeutic agents exhibit their effect by preventing alveolar damage and inhibiting accumulation of inflammatory cells in the pulmonary tissues [16]. In the present study, RSV infection caused a marked damage to the pulmonary tissues and increased accumulation of inflammatory cells. However, bryostatin1 pre-treatment of the RSV infected mice suppressed inflammatory cell aggregation in the pulmonary tissues. In the animal models of pneumonia host-virus interaction leads to rapid and massive inflammatory cytokine recruitment in the pulmonary tissues [20]. In the current study RSV infection markedly enhanced secretion of IL-1β, -18 and TNF-α in the pulmonary tissues as well in BALF. Treatment of the RSV infected mice with bryostatin 1 suppressed the secretion of inflammatory cytokines markedly when compared to untreated mice. These findings provide evidence that bryostatin 1 inhibits RSV infection in the mice through suppression of inflammatory cytokine generation.

Activation of NLRP3 inflammasome following host-virus interaction has been reported to play crucial role in the development of pneumonia and its inhibition is considered to be of significance for the treatment of pneumonia [12,21]. Studies have shown that NLRP3 activation causes caspase-1 cleavage as well as its activation which subsequently stimulates inflammatory cytokine generation and development of inflammation [22,23]. In the present study, RSV infection significantly induced NLRP3, ASC and caspase-1 activation in the mice model. Treatment of the mice with bryostatin 1 caused down-regulation of RSV induced activation of NLRP3.

## CONCLUSION

The findings of this study demonstrate that bryostatin 1 pre-treatment leads to a marked reduction in the viability of RSV-infected BEAS-2B cells and prevents RSV-induced pneumonia in mice. Pre-treatment of RSV infected mice with bryostatin1 prevented pulmonary tissue damage through suppression of inflammatory cytokine generation and down-regulation of NLRP3 activation. Therefore, bryostatin 1 may be used for the development of an effective treatment strategy for pneumonia.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aili Zhang designed the study and wrote the paper. Feng Xu, Jun Chen, Beizheng Xu, Haiyan Liu and Jian Song performed the experimental work, carried out the literature study and compiled the data. All the authors performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors before communication for the consideration of publication.

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