# Oxidative stress modulates the expression of toll-like receptor 3 during respiratory syncytial virus infection in human lung epithelial A549 cells

MIN-MIN WANG[1*], MIN LU[2*], CHUAN-LONG ZHANG[3*], XUAN WU[1], JING-XIAN CHEN[1,4], WEI-WEI LV[1], TAO SUN[1], HUAN QIU[1] and SHENG-HAI HUANG[1,5]

[1]Department of Microbiology, School of Basic Medical Sciences, Anhui Medical University; [2]Department of Laboratory, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230032; [3]Department of Pediatrics, The People's Hospital of Lu'an City, Lu'an, Anhui 237005, P.R. China; [4]Department of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA; [5]Department of Bioengineering, School of Life Sciences, Anhui Medical University, Hefei, Anhui 230032, P.R. China

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**Abstract.** Toll-like receptor 3 (TLR3) can react with double stranded RNA and is involved in the inflammatory response to respiratory syncytial virus (RSV) infection. Also, oxidative stress has been reported to be involved in RSV infection. However, the correlation between oxidative stress and TLR3 activation during RSV infection is unclear. Therefore, the present study investigated the association between TLR3 expression and oxidative stress modulation during RSV infection in A549 cells. For comparison, seven treatment groups were established, including RSV-treated cells, N-acetyl-L-cysteine (NAC)+RSV-treated cells, oxidant hydrogen peroxide ($H_2O_2$)+RSV-treated cells, normal cell control, inactivated RSV control, NAC control and $H_2O_2$ control. The mRNA expression changes of TLR3, interferon regulatory factor-3 (IRF3), nuclear factor-κB (NF-κB) and superoxide dismutase 1 (SOD1) were measured using semi-quantitative reverse transcription-polymerase chain reaction, and the protein changes of TLR3 and phospho-NF-κB p65 were determined using western blot in A549 cells from the different treatment groups. The present study also evaluated the differences in hydroxyl free radical (·OH), nitric oxide (NO) and total SOD activity in the different treatment groups. The results demonstrated that RSV infection of A549 cells increased the levels of ·OH and NO, while decreasing the activity of total SOD. Pretreatment of A549 cells with $H_2O_2$ prior to RSV infection upregulated the mRNA and protein expression of TLR3 and NF-κB, and downregulated the mRNA expression of IRF3 and SOD1, as well as the total SOD activity. When the infected cells were pretreated with NAC, the mRNA and protein expression of these genes were reversed. These variations in the TLR3-mediated signaling pathway molecules suggested that oxidative stress may be a key regulator for TLR3 activation during RSV infection. RSV-induced oxidative stress may potentially activate TLR3 and enhance TLR3-mediated inflammation. These results may provide better understanding of the RSV-induced inflammatory and immune pathways, and may also contribute to the drug development and prevention of human RSV diseases.

*Correspondence to:* Professor Sheng-Hai Huang, Department of Microbiology, School of Basic Medical Sciences, Anhui Medical University, 81 Meishan Road, Shushan, Hefei, Anhui 230032, P.R. China
E-mail: huangshh68@aliyun.com

*Contributed equally

*Abbreviations:* dsRNA, double stranded RNA; GSH, glutathione; $H_2O_2$, hydrogen peroxide; IFN, interferon; IRF3, interferon regulatory factor-3; ISRE, IFN-stimulated response element; MDA, malondialdehyde; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; NS, nonstructural protein; ·OH, hydroxyl free radical; PAMPs, pathogen-associated molecular patterns; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; RSV, respiratory syncytial virus; SOD, superoxide dismutase; TLRs, Toll-like receptors; TLR3, Toll-like receptors 3; pi, post infection; RT, room temperature

*Key words:* A549 cells, oxidative stress, respiratory syncytial virus, toll-like receptor 3

## Introduction

Respiratory syncytial virus (RSV), a single-stranded negative-sense RNA virus (1), not only causes severe lower respiratory tract infections in infants worldwide but also leads to hospitalization of infants (2-4). As one of the main targets for RSV infection, airway epithelial cells play a very important role in host defense system against RSV infection. Recently, cultures of human respiratory epithelium have been applied to study the mechanisms of RSV infection (5). After RSV infection, airway epithelial cells produce a large number

of immune-active molecules, including cytokines, chemokines and reactive oxygen species (ROS) to promote the occurrence of infantile bronchiolitis and pneumonia (6).

Oxidative stress is an invariable feature of human lung epithelial cells, resulting in a large amount of ROS productions that modifies and disrupts cellular biomolecules during immune-inflammatory responses to viral infection (7). This injury is mainly the results of additional ROS produced by further increased oxidative stress (8). It has been reported that RSV could induce the RelA activation mediated by ROS signaling (9). The elevation of ROS and IRF3 signals caused by RSV infection in airway epithelial cells could be blocked by anti-oxidants (10). The formation of ROS produces an imbalance between the antioxidant defenses and oxidant molecules such as hydroxy radicals, hydrogen peroxide, and superoxide anion radicals. ·OH provides strong evidence of increased oxidative stress and is an effective initiator of highly reactive lipid peroxidation (11,12). In addition, nitric oxide (NO) is a key mediator for airway inflammation, which promotes the migration of inflammatory cells to the airway (12,13). ROS and free radicals have been shown to act as cellular signaling molecules participating in various molecular and biochemical processes, including pro-inflammatory mediations such as chemokines and cytokines expressions (14). RSV-induced intracellular ·OH and NO may therefore modulate the expression of pro-inflammatory mediators, and oxidative stress may represent an important mechanism for RSV-induced lung pathogenesis. On this basis, Mastronarde *et al* (15) proposed that antioxidants might be able to block IL-8 production following RSV infection *in vivo*. In contrast, the activities of antioxidant enzymes (e.g. SOD, glutathione peroxidase, catalase, and glutathione S-transferase) are very important for cellular defense against RSV infection in A549 cells (6). Our group previously revealed that RSV-intranasally-inoculated mice can react to oxidative stress by increasing the malondialdehyde (MDA), NO and ·OH levels, and reducing SOD and GSH activities in lung tissues. The application of melatonin with anti-oxidant and anti-inflammatory functions reversed the pathophysiology by reducing lung inflammation and ameliorated clinical presentations in RSV-infected mice (16), suggesting that oxidative stress is involved in RSV infection.

Toll-like receptors (TLRs) play a fundamental role in human innate anti-microbial immunity and inflammations by recognizing the conserved pathogen-associated molecular patterns (PAMPs) (17,18). Among them, toll-like receptor 3 (TLR3) and TLR7 are considered as the main mediators of viral-induced signal transductions. TLR3, for example, is able to identify double-strand viral genomic RNA and the replicative intermediates of RSV (19), suggesting that TLR3 plays a role in resisting RSV infection in human respiratory system (20). The reaction of TLR3 with dsRNA activates intracellular signaling and promotes the biosynthesis and secretion of cytokines and other inflammatory mediators. Dou *et al* (21) reported that RSV induced gene expression of TLR3 and TNF-α both *in vitro* and in mouse lungs. Once TLR3 is activated, its downstream signaling pathway will lead to the activation of nuclear factor-κB (NF-κB) and interferon regulatory factor-3 (IRF3) (22). NF-κB has been shown to modulate the production of pro-inflammatory cytokines, such as IL-1β, TNF-α and the neutrophil chemoattractant IL-8 (23), which are strongly associated with the outcome of inflammatory disease. Whereas IRF3 was shown to regulate the type I interferon (IFN) expressions (24). Recently, an increase in TLR3 expression was observed in airway epithelial cells of patients with acute respiratory distress syndrome under airway exposure to hyperoxic conditions (25), enhanced TLR3 responses to oxidative stress have also been found in airway epithelial cells (26). These phenomena suggest that oxidative stress may participate in the regulation of TLR3 expression.

N-acetyl-L-cysteine (NAC) is a thiol compound that directly used as a free radical scavenger and a reduced glutathione (GSH) precursor (27), which allow it to be used as an antioxidant in a broad spectrum (28). On the contrary, hydrogen peroxide ($H_2O_2$) induces oxidative and inflammatory responses in epithelial cells and it can be used as an oxidant (29).

To our knowledge, by now, there is no study reporting that whether RSV infection increases TLR3 signaling in airway epithelial cells through oxidative stress induction. In order to understand the relationship between TLR3 expression and oxidative stress modulation during RSV infection in A549 cells, we studied the intervening effects of oxidative stress agonist hydrogen peroxide ($H_2O_2$) and inhibitor N-acetyl-L-cysteine (NAC) on TLR3 expression. Besides, we proposed that oxidative stress induced by RSV infection might serve as one of the key events in the process of TLR3 activation We hoped that our study would provide a potential new pharmacological method to improve RSV-induced acute lung inflammation.

## Materials and methods

*Cells, viruses and reagents*. The human lung adenocarcinoma alveolar basal epithelial cell line A549 (ATCC® CCL-185®; American Type Culture Collection, Manassas, VA, USA) was gifted by Professor Hai-Ming Wei, Institute of Immunology, University of Science and Technology of China (USTC, Hefei, Anhui, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin (100 U/ml)-streptomycin (100 μg/ml) at 5% $CO_2$ and 37˚C.

The laryngeal epithelial carcinoma HEp-2 cell line was maintained in our laboratory. Though it has been reported being contaminated by HeLa cells, an epithelium-like cell from a cervical adenocarcinoma (iclac.org/databases/cross-contaminations/), Nevertheless, HEp-2 cells are still a good substrate for RSV (30). Additionally, RSV used in this study was harvested from culture supernatant and was further purified by density gradient centrifugation; therefore, either HEp-2 or HeLa cells would have no intervening effect on the result interpretation of the analysis.

The RSV Long strain was also gifted by Professor Hai-ming Wei, Institute of Immunology, University of Science and Technology of China (USTC, Hefei, Anhui, China) and was multiplicated in HEp-2 cells. Then, the culture supernatant was precipitated by polyethylene glycol 4000, followed by centrifugation on 35-65% discontinuous sucrose gradients. Purified virus suspension was aliquoted, quickly frozen, and stored in liquid nitrogen. The viral titer of purified RSV reached $7x10^6$ PFU/ml as measured by a methylcellulose plaque assay in HEp-2 cells (31).

The UV-inactivated RSV was prepared as follows: A one ml RSV pool was transferred to a culture plate. The plate was placed under a germicidal lamp TUV-15 W/G15 T8 (Philips, The Netherlands) and irradiated at a distance of 10 cm in 3-min intervals, with swirling between the intervals, for a total of 30 min. UV-inactivated virus titers were also determined by plaque assays on HEp-2 cells to confirm the inactivation effect, then the virus stock was stored in liquid nitrogen until use.

$H_2O_2$ and NAC were purchased from Sigma Corporation (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Commercial assay kits for measuring ·OH, NO and SOD activities were purchased from Nanjing Jiancheng Bioengineering Institute, (Nanjing, China).

*Experimental design and sample collection*. Seven experimental groups were assigned: normal groups (untreated and uninfected with RSV); $H_2O_2$ control groups (pretreated with $H_2O_2$ without RSV infection); NAC control groups (pretreated with NAC without RSV infection); RSV infection control (cells infected with RSV but no pretreatment); NAC+RSV groups (pretreated with NAC at 5 mM prior to RSV infection), $H_2O_2$ +RSV groups (pretreated with $H_2O_2$ at 150 $\mu$M prior to RSV infection) and inactivated RSV groups (cells infected with inactivated RSV but no pretreatment). The pretreatment time with $H_2O_2$ or NAC for the corresponding group was 1 h. Cells in the corresponding groups were infected with RSV at MOI=1 in serum-free mediums for 2 h before fresh mediums were added. The culture supernatants and trypsinized cells were separately collected by centrifugation at 4, 8, 12 and 24 h post infection (pi) and the samples were assessed by the following experiments.

*Measurement of ·OH and NO in culture supernatants*. ·OH concentration was determined using a commercial ·OH assay kit based on the Fenton reaction method according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute) (32). Briefly, the reaction mixtures were added to a quartz capillary tube, then Griess chromogenic reagent was added to form a colored substance for another 20 min at room temperature (RT) (16). The color depth of the substance is proportional to the amount of ·OH (33). The absorbance values at 550 nm were recorded, and the data was expressed as units/ml.

NO concentration was determined using a commercial NO assay kit based on the Griess reaction method according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute) (34). First, 50 $\mu$l of Griess reagent I was added to 100 $\mu$l of the supernatant from A549 cultures. Second, 50 $\mu$l of Griess reagent II was added. Third, the reaction mixture was incubated for 10 min at RT, and 160 $\mu$l of the supernatant was used for detection after centrifugation at 3500 rpm for 15 min. Last, the absorbance at 550 nm was recorded, and the results were expressed as $\mu$mol/l. All samples were tested in triplicate.

*Measurement of total SOD activity in A549 cells*. Proteins from A549 cells were prepared as a method previously described (16). First, the cells were washed with PBS before the treatment with Versene-Trypsin PBS solution. Then, the trypsin-digested cells were centrifuged at 1,400 g for 10 min and the pellet was re-suspended in a lysis buffer supplemented with protease inhibitors. Next, after 30 min of incubation on the ice, the extraction mixture was centrifuged at 12,000 g at 4˚C for 30 min. Last, the supernatant was transferred to a fresh tube, and its protein concentration was measured by the Lowry method.

Superoxide dismutase (SOD), an important enzyme in the antioxidant system, is able to convert $O_2^-$ to hydrogen peroxide ($H_2O_2$) and eventually change them to water. The mechanism for total SOD assay was based on its ability to inhibit the oxyamine oxidation within the xanthine/xanthine oxidase system (35). SOD activity was determined using a commercial WST-1 assay kit (Nanjing Jiancheng Bioengineering Institute). The absorbances at 450 nm at the reaction endpoint were read by a microplate reader (ELX800UV; BioTek Instruments, Inc., Winooski, VT, USA). The SOD activity of each sample was calculated using a previously described equation (36). All samples were tested for three times, and the results were expressed as units per mg protein.

*TLR3, NF-κB p65, IRF3 and superoxide dismutase 1 (SOD1) mRNA semi-quantification by reverse transcription polymerase chain reaction (RT-PCR)*. The kinetics of gene expression of *TLR3*, *NF-κB p65*, *IRF3* and *SOD1* were analyzed by semi-quantitative RT-PCR. Total RNA was extracted from A549 cells with TRI Reagent™ (Sigma-Aldrich; Merck KGaA) following the manufacturer's instructions. Then, the total RNA was treated with RNase-free DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) to remove genomic DNA contamination and reverse-transcribed into cDNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis kit (Fermentas; Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Next, the synthesized first strand cDNA was amplified by PCR with the primers for TLR3, NF-κB p65, IRF3, or SOD1 genes. The PCR primers (Table I) for TLR3, NF-κB p65, IRF3, SOD1 and β-actin genes were designed using the Primer Express software, as previously described (37). The reaction conditions and cycle numbers used for the PCR of each gene were shown in Table II. PCR products and a DNA molecular weight marker (DL2000; Takara Biotechnology Co., Ltd., Dalian, China) were electrophoresed in a 1.5% agarose gel and visualized under UV light. The density of the bands was quantified by densitometry using Labworks software, and the expression levels were expressed as the fold-increase compared to β-actin controls.

*TLR3 and p-NF-κB protein quantification by western blot*. The protein level changes of TLR3 and p-NF-κB were analyzed by western blot assay. First, the cells in each group were washed with PBS solution and lysed in a lysis buffer containing phenylmethylsulfonyl fluoride (PMSF). All samples were incubated on ice for 30 min and centrifuged at 11,000 g for 5 min. Second, the protein concentrations in each group of centrifuged supernatant were determined by the Lowry method. Third, fifty $\mu$g of protein was run on a 10% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (sc-296042; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Fourth, nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 2 h at RT. Fifth, the membranes were incubated with rabbit anti-TLR3 antibody (sc-28999; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and anti-p-NF-κB antibody (sc-33020; Santa Cruz

Table I. Primer sequence and length for polymerase chain reaction products of each human gene.

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | Size (bp) |
|---|---|---|---|
| TLR3 | GATCTGTCTCATAATGGCTTG | GACAGATTCCGAATGCTTGTG | 304 |
| NF-κB p65 | CACAAGGAGACATGAAACAG | CCAGAGACCTCATAGTTGT | 187 |
| SOD1 | ATGGCGACGAAGGCC | TTATTGGGCGATCCC | 465 |
| IRF3 | GACCTCACGACCCACATAA | ACCCCACCAGCCGCAGGCCC | 377 |
| β-actin | GGCTTTGAGTAATGAGAATTTCGA | ATCAGTTGCAATCAAGAAGTGTTG | 520 |

TLR4, Toll-like receptor 3; NF-κB p65, nuclear factor-κB p65; SOD1, superoxide dismutase 1; IRF3, interferon regulatory factor-3.

Table II. Polymerase chain reaction thermocycling conditions and cycle numbers for each gene.

| Gene | Initial Denature | Denature | Annealing | Extension | No. of cycles |
|---|---|---|---|---|---|
| TLR3 | 94˚C for 5 min | 94˚C for 40 sec | 55˚C for 45 sec | 72˚C for 1 min | 32 |
| NF-κB p65 | 94˚C for 5 min | 94˚C for 40 sec | 57.7˚C for 45 sec | 72˚C for 1 min | 32 |
| SOD1 | 94˚C for 5 min | 94˚C for 40 sec | 57˚C for 45 sec | 72˚C for 1 min | 32 |
| IRF3 | 94˚C for 5 min | 94˚C for 40 sec | 49.4˚C for 45 sec | 72˚C for 1 min | 32 |
| β-actin | 94˚C for 5 min | 94˚C for 40 sec | 55˚C for 45 sec | 72˚C for 1 min | 32 |

TLR4, Toll-like receptor 3; NF-κB p65, nuclear factor-κB p65; SOD1, superoxide dismutase 1; IRF3, interferon regulatory factor-3.

Biotechnology Inc.) separately at a 1:1,000 dilution overnight at 4˚C. After washing with TBST for 3x10 min, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) (sc-2004; Santa Cruz Biotechnology Inc.) for 2 h at RT. Last, the blots were washed, and the antigens were visualized using an enhanced chemiluminescence western blot detection system (SuperSignal West Femto kit; Thermo Scientific, Inc.) and recorded by a Tanon 4500 automatic digital gel image analysis system (Tanon 4500; Tanon, Shanghai, China).

A β-actin monoclonal antibody (1:1,000; TA-09; OriGene Technologies, Inc., Beijing, China) and horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10,000; sc-2005; Santa Cruz Biotechnology Inc.) were used as the internal control, The molecular weights of TLR3, NF-κB and β-actin in the PAGE gel were 104, 65 and 43 KDa, respectively. The density of the bands on the blot was quantified using Labworks imaging software. Compared with β-actin control group, densitometry in each group was expressed as the fold-increase.

*Statistical analysis*. The resulting data are recounted as the mean ± standard error mean. One-way analysis of variance analysis and the Fisher post hoc test were performed to determine statistical significance among each group using SPSS v.17.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ for the null hypothesis was considered to indicate a statistically significant difference.

## Results

*The releasements of ·OH and NO after different treatments*. Oxidative stress can be induced from either excess ROS generation or impaired antioxidant capacity, which produced free radicals and reactive oxygen molecules in activated cells (11). Because that oxidative stress-mediated events were correlated with the releasement of ·OH and NO, therefore, ·OH generation was considered as a marker of free oxygen species due to the extreme instability of ROS. In this study, the NO and ·OH in A549 cells from inactivated-RSV control, NAC control and $H_2O_2$ control groups, at different time points, had no significant changes compared to the normal control group within 24 h (Fig. 1). However, in both the RSV-infected group and the $H_2O_2$+RSV-treated group (Fig. 1A), the ·OH concentration increased dramatically compared to the normal cell control group ($P<0.01$) Nevertheless, in cell group treated with NAC+RSV (Fig. 1A), although the ·OH levels also increased with an increasing time of RSV infection, they were significantly lower than those in the RSV-infected group at the corresponding time points. Thus, the ·OH elevations were associated with the extended time duration of RSV infection, suggesting ·OH in A549 cells was up-regulated in a time-dependent manner after RSV infection. On the results of NO concentration, its variation had a similar change pattern to that of ·OH concentration (Fig. 1B), which also demonstrated that the RSV infection induced a significant elevation of oxidative stress in A549 cells. Notably, UV-inactivated RSV did not elevate the ·OH and NO levels compared with the normal group (data not shown), suggesting that the alteration of oxidative stress is dependent on virus replication.

*The total SOD activity after different treatments*. The protein level and activity of SOD are important indicators for the cellular antioxidant stress capacity, and increased SOD expression can

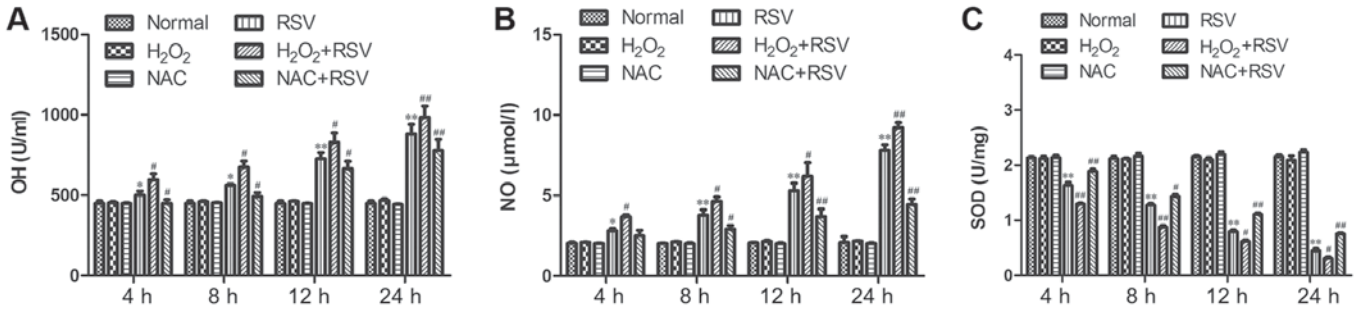


Figure 1. Production of ·OH and NO in culture supernatants, and total SOD activity in cell lysates in each group following different treatments.·OH was measured by (A) Fenton reaction, and NO was determined by (B) Griess reaction in each group at various time points. The concentration of ·OH was significantly different among the RSV-treated group and $H_2O_2$+RSV group or NAC+RSV group at 4 h pi. The level of NO was also significantly different among the RSV-treated group and $H_2O_2$+RSV group or NAC+RSV group at 8 h pi. (C) The total SOD activity was assessed by a commercial assay kit for the determination of total SOD based on the hydroxylamine method. When compared with the normal cell group, the total SOD activity in the RSV-treated group was substantially decreased at 4, 8, 12 and 24 h pi. A significant decrease was also observed in the $H_2O_2$+RSV-treated group compared with the RSV-treated group. However, SOD activity was markedly reversed in the NAC+RSV-treated group when compared with the RSV-treated group and $H_2O_2$+RSV-treated group. Data are expressed as the mean ± standard error mean of three independent experiments. $^{*}$P<0.05 and $^{**}$P<0.01 vs. normal cell control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. RSV-treated group. ·OH, hydroxyl free radical; NO, nitric oxide; pi, post infection; SOD, superoxide dismutase; RSV, respiratory syncytial virus; NAC, N-acetyl-L-cysteine; $H_2O_2$, hydrogen peroxide.

contribute to antioxidant functions *in vivo* (16). As shown in Fig. 1C, the SOD levels were significantly decreased in a time-dependent manner in the RSV-infected group compared with those in the normal group at all time points tested (P<0.01; Fig. 1C). In contrast, although pretreatment of A549 cells with inactivated-RSV or NAC or $H_2O_2$ alone had no impact on SOD release within 24 h, the administration of NAC+RSV, but not $H_2O_2$+RSV, was able to markedly improve SOD activity during infection compared with RSV-infected group (Fig. 1C). Thus, the antioxidant capacity of RSV-infected A549 cells was enhanced by the pretreatment of NAC, which suggested that oxidative stress damage indeed occurred after RSV infection.

*The mRNA levels of TLR3, NF-κB p65, IRF3 and SOD1 genes after different treatments*. TLR3 activation and the mRNA expression levels of its downstream molecules, including NF-κB p65, IRF3 and SOD1, were analyzed by semi-quantitative RT-PCR in this study. Figs. 2 and 3 showed the mRNA expression of TLR3, NF-κB p65, SOD1 and IRF3 in A549 cells with RSV infection (Figs. 2A and 3A) and cultures pretreatment of $H_2O_2$ (Figs. 2D and 3B) or NAC (Figs. 2E and 3C). The results showed that adding inactivated-RSV (data not shown) or $H_2O_2$ (Fig. 2B) or NAC (Fig. 2C) alone has no effect on TLR3 mRNA expression in A549 cells. However, the RSV infection treatment and $H_2O_2$+RSV treatment significantly elevated both TLR3 (Fig. 2F) and NF-κB p65 (Fig. 3D) mRNA levels at all time points tested. Conversely, the mRNA expression of TLR3 and NF-κB was decreased in the NAC+RSV group compared to the RSV group, which suggested that NAC played an inhibitory role in RSV-induced TLR3 and NF-κB activation.

In RSV-treated and RSV+$H_2O_2$-treated groups, the levels of IRF3 and SOD1 mRNA were markedly decreased with statistically significant differences, compared to the normal cell group (Fig. 3). This reduction could be reversed by the NAC+RSV treatment, but the mRNA levels of IRF3 and SOD1 in NAC+RSV-treated group were still less than that shown in the RSV-treated group.

Therefore, the RSV infection in A549 cells enhanced the mRNA expression of TLR3 and NF-κB p65 but suppress the mRNA expression of IRF3 and SOD1, indicating that the oxidative stress inhibitor and agonist altered TLR3 activation and the expression of relative downstream signaling molecules at the transcriptional level. These data are in line with the results described above with the determination of ·OH and NO concentration and total SOD activity.

*The protein expression of TLR3 and p-NF-κB after different treatments*. Because that TLR3 was recently reported to be able to recognize the viral dsRNA intermediates produced during RSV replication and activate NF-κB (20,22), the expression of both TLR3 and p-NF-κB proteins were studied by western blot assay. It was found that there were no significant differences in TLR3 and p-NF-κB changes in inactivated-RSV control or $H_2O_2$ control or NAC control groups at the indicated concentrations (Fig. 4), therefore the use of inactivated-RSV or $H_2O_2$ or NAC alone had no effect on the experimental results. As shown in Fig. 4, TLR3 and p-NF-κB protein were significantly increased in the RSV-treated and the $H_2O_2$+RSV-treated groups compared to the normal cell control group. However, the treatment with NAC+RSV attenuated the RSV-induced elevation of TLR3 and p-NF-κB protein, suggesting that NAC may inhibit RSV-induced TLR3 activation (Fig. 4F).

These results demonstrate that the elevated TLR3 expression is consistent with the increasing of p-NF-κB protein expression during RSV infection, and the TLR3 activation can enhance the up-regulation of its downstream signaling proteins, including NF-κB.

## Discussion

RSV is a highly pathogenic virus that can lead to severe respiratory diseases in newborns, children, the elderly and individuals with immune impairment (38,39). It has been reported that airway inflammation plays a crucial role in the disease outcome in RSV-infected hosts (40). Although the pathogenesis of RSV infection remains largely unknown, previous studies have suggested that a relative overload of

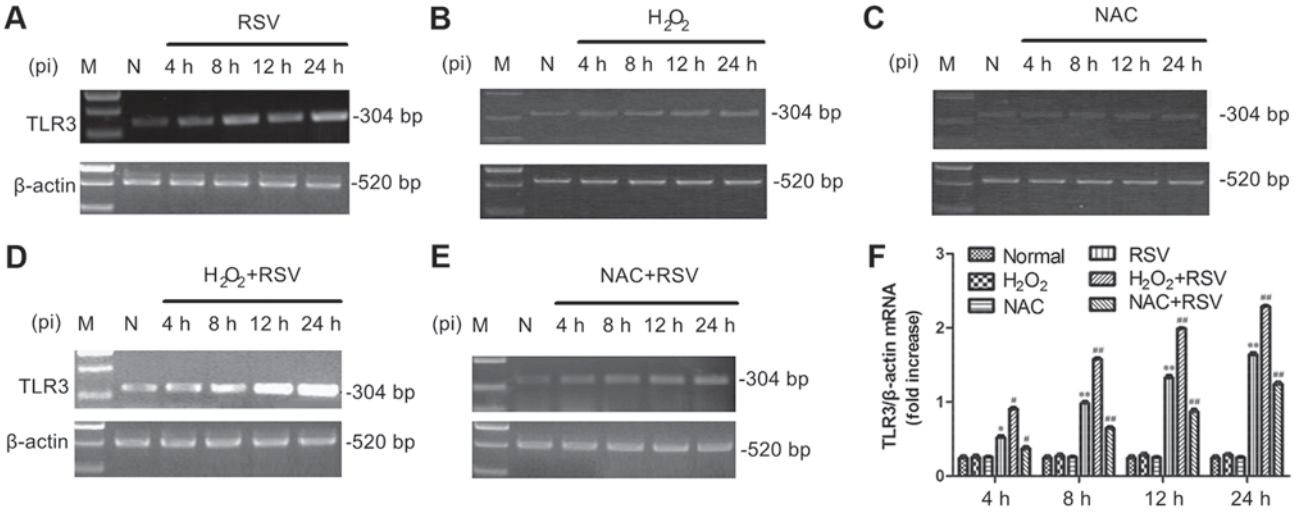


Figure 2. TLR3 mRNA expression levels in A549 cells of each group following different treatments. This combined figure presents the changes of TLR3 mRNA expression levels detected by semi-quantitative reverse transcription-polymerase chain reaction assay at various time points in the (A) RSV-treated group, (B) $H_2O_2$-treated group, (C) NAC-treated group, (D) $H_2O_2$+RSV-treated group and (E) NAC+RSV-treated group. The normal cell group, (B) $H_2O_2$-treated group and (C) NAC-treated group were included as control groups. It was revealed that there was no statistical difference in TLR3 mRNA expression levels among each control group. (F) The relative content of TLR3 gene transcription was calculated using Labworks software and expressed as fold increase. Data are expressed as the mean ± standard error mean of three independent experiments. $^{*}$P<0.05 and $^{**}$P<0.01 vs. normal cell control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. RSV-treated group. TLR3, Toll-like receptor 3; $H_2O_2$, hydrogen peroxide; NAC, N-acetyl-L-cysteine; RSV, respiratory syncytial virus; pi, post infection.

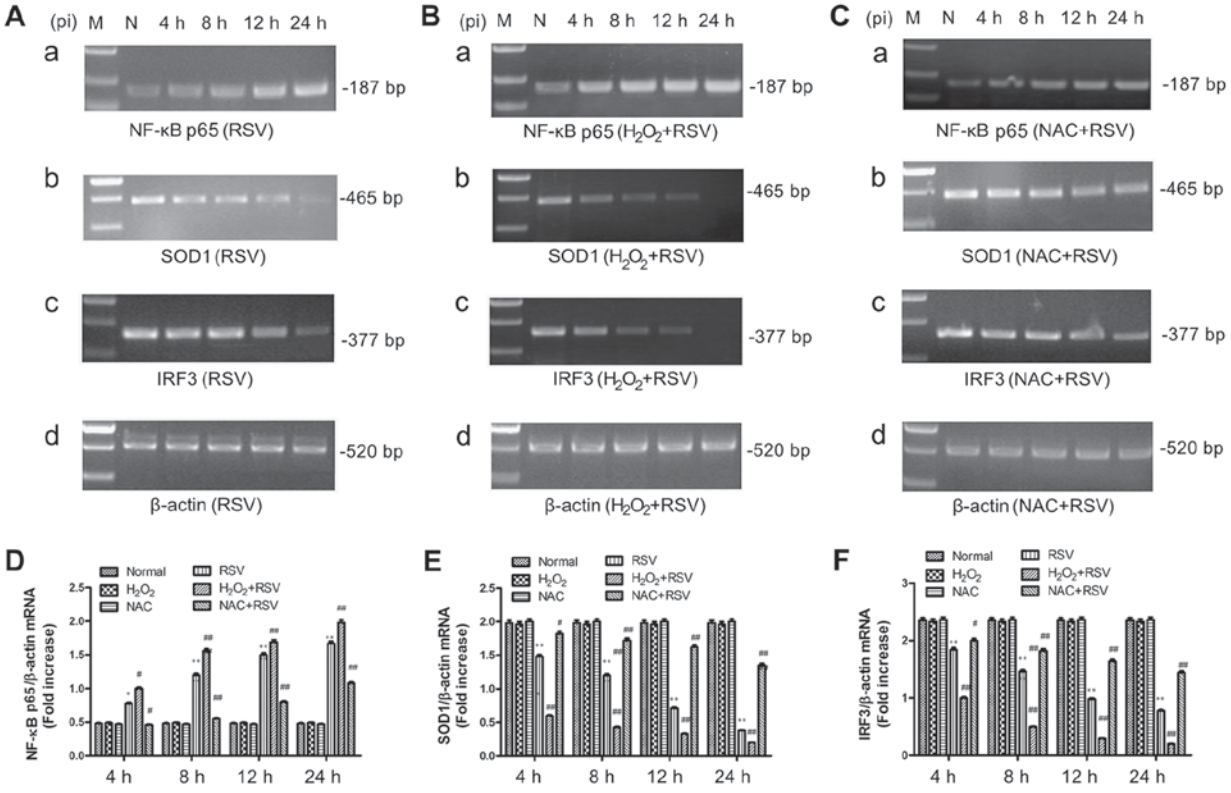


Figure 3. mRNA expression levels of NF-κB p65, SOD1 and IRF3 in each group were measured by semi-quantitative verse transcription-polymerase chain reaction assay. Differences in the mRNA expression of NF-κB p65, SOD1, IRF3 and β-actin genes were measured in the (A) RSV-treated group, (B) the $H_2O_2$+RSV-treated group and (C) the NAC+RSV-treated group, respectively. The relative content of mRNA levels for (D) NF-κB p65, (E) SOD1 and (F) IRF3 genes were quantified and calculated using Labworks software. The unit of densitometry was expressed as fold increase. Data are expressed as the mean ± standard error mean of three different experiments groups. $^{*}$P<0.05 and $^{**}$P<0.01 vs. normal cell control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. RSV-treated group. NF-κB p65, nuclear factor-κB p65; SOD, superoxide dismutase; IRF3, interferon regulatory factor-3; pi, post infection; RSV, respiratory syncytial virus; $H_2O_2$, hydrogen peroxide; NAC, N-acetyl-L-cysteine.

oxidants in response to RSV infection in human airway epithelial cells may have an important impact on lung injury (41).

Oxidative stress was reported to induce the production of reactive oxygen species (ROS), causing oxidative damage in

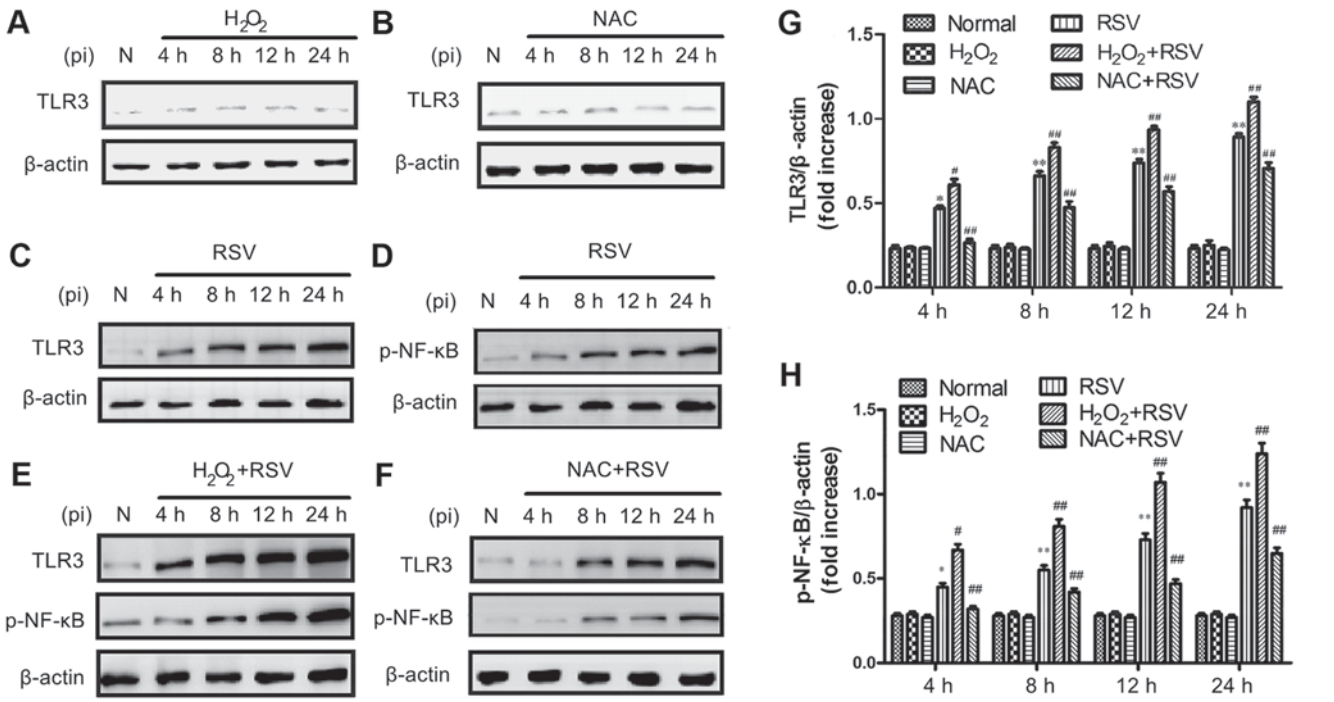


Figure 4. Protein expression levels of TLR3 and p-NF-κB were assessed by western blot analysis. There was no difference in TLR3 production observed in the only (A) $H_2O_2$-treated or only (B) NAC-treated control groups. (C) TLR3 and (D) p-NF-κB activation were enhanced with the extended time duration following RSV infection. (E) There was an increase in TLR3 and p-NF-κB protein production in the $H_2O_2$+RSV-treated group. (F) In addition, an increase in TLR3 and p-NF-κB protein expression was observed in the NAC+RSV-treated group; the increase was less than that shown in the RSV-treated group. The ratio changes of (G) TLR3 and (H) p-NF-κB density were determined using Labworks software and analyzed using GraphPad Prism 5 software. Data were obtained from three independent experiments and are presented as the mean ± standard error mean. $^{*}$P<0.05 and $^{**}$P<0.01 vs. normal cell control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. RSV-treated group. TLR3, Toll-like receptor 3; NF-κB, nuclear factor-κB; pi, post infection; $H_2O_2$, hydrogen peroxide; NAC, N-acetyl-L-cysteine; RSV, respiratory syncytial virus.

tissue during RSV infection *in vivo* (11,42). Also, the decrease of SOD total activity can result in an excess availability of superoxide and generate hydroxyl radicals that are associated with the initiation and propagation of lipid peroxidation (43). Though a study has reported that RSV infection of A549 cells was able to induce a significant decrease in SOD1, SOD3, GST and catalase gene expressions along with the increase of SOD2; however, the total SOD activity would decrease initially, followed by a subsequent increase after 24 h (41). The latter observations are consistent with our findings in the current study, namely that total SOD activity is decreased within 24 h of RSV infection.

The activation of TLR2, TLR3, TLR4, and TLR7/8 in the innate immune system can lead to the strong up-regulation of SOD2 gene expression in macrophages during microbial infection (44). To *et al* (45) had demonstrated that influenza A virus-induced TLR7 activation enhanced the oxidative burst of NOX2-oxidase dependence in macrophages, suggesting the occurring of acute lung injury after influenza A virus infections. Although oxidative stress was recently found to augment the response of TLR3 to dsRNA in airway epithelial cells through NF-κB pathway (26), to date, there is no study described the relationship between RSV infection with both TLR3 activation and oxidative stress generation, therefore, we performed the experiments and firstly reported that the elevated expression of TLR3 could be modulated by oxidative stress during RSV infection.

TLR3 gene expression may be regulated by RIG-I-induced IFN-β secretion in the early response of host cells to RSV infection and TLR3 indeed mediates epithelial responses to RSV infection (46). Our group previously found that RSV infection induced TLR gene transcription by recognizing the viral dsRNA genome, and it is likely that RSV infection promotes a rapid activation of innate responses via the increased expression of TLR3 (47). However, the molecular mechanism for RSV-induced TLR3 up-regulation was unclear (48,49). In the present study, RSV infection was shown to up-regulate both mRNA and protein expression levels of TLR3 in A549 cells, furthermore, it was also shown that RSV-induced, TLR3-mediated early signal events could lead to the activation of NF-κB and IRF3, both of which were two key transcriptional factors for the expression of inflammatory cytokines and chemokines in airway epithelial cells. Pretreatment with $H_2O_2$ before RSV infection increases TLR3 expression and TLR3-mediated NF-κB activity, whereas pretreatment with antioxidant NAC inhibits the activation of TLR3 pathways, including pNF-κB expression (Figs. 2-4). These results are in agreement with our previous finding that RSV can induce TLR3 expression and NF-κB/RelA subunit phosphorylation and transcriptional activation (30).

Matsukura *et al* (50) reported that dsRNAs such as poly (I:C) bound to TLR3 that distributed on the cell surface and induced some chemokines and cytokines gene expressions through activation of NF-κB and IRF3. Their results indicated that dsRNA could increase the expression of inflammatory cytokines and chemokines via TLR3-NF-κB and TLR3-IRF3 signaling in airway epithelial cells (50,51). Liu *et al* (46)

showed that the TLR3 knockdown mediated by siRNA significantly reduced NF-κB/RelA transcriptional levels by blocking the activating phosphorylation of NF-κB/RelA at serine residue 276. It was also demonstrated that NADPH oxidase 2 (NOX2) mediates ROS production in RSV-infected human airway epithelial cells and that NOX2 acts upstream of the phosphorylation of both IκBα at $Ser^{32}$ and of p65 at $Ser^{536}$ in RSV-infected A549 cells and in human bronchial epithelial cells (52). Jamaluddin *et al* (9) demonstrated the RSV-induced ROS induced activation of RelA and RSV-induced ROS formation also resulted in STAT activation and IRF gene expression induction (10). Unanimously, our current study revealed that oxidative stress was involved in RSV-augmented NF-κB and IRF activation in airway epithelial cells and lead to an increase of TLR3 expression.

It is worth noting that preferential induction of TLR3 is also observed in human astrocytes after its exposure to $H_2O_2$ to induce oxidative stress (53) and that $H_2O_2$, but not poly (I:C), appears to activate NF-κB and nuclear translocation of p65 in human SH-SY5Y neuroblastoma cells (54). ROS may potentiate TLR3 expression through NOX2 signaling by RSV infection (55).

As far as the investigations are concerned, we further confirmed that there were no significant differences in TLR3 signaling in A549 cells pretreated with either $H_2O_2$ or NAC without RSV infection (Fig. 2B, C and Fig. 4A, B), as previously described (26,56). Geiler *et al* (56) reported that there was no significant decrease in virus titer produced in A549 cells as a result of NAC treatment (5 mM) at 48 h pi with influenza A virus; thus, we chose to use NAC at 5 mM in the current study. Unfortunately, this concentration of NAC did not fully recover SOD activity to the normal levels, nor did we detect the effect of $H_2O_2$ on virus titer, both of which are the limitations of the present study.

The transcriptional activators of the NF-κB family participated in the modulation of cell proliferation, differentiation, apoptosis, inflammation, immunity and cytokine expression in response to various types of stimulation (57). In the inactive state, the NF-κB is mainly located in the cytoplasm, forming complexes with IκB. Once it is stimulated by extracellular stimuli such as viruses, IκB can be rapidly phosphorylated and degraded, allowing the release of NF-κB p65 and subsequent NF-κB p65 translocation to the nucleus with the increase of NF-κB regulated gene expression (58). IRF3 acts as an important transcriptional regulator in antiviral immune responses, viral infection can also induce the IRF3 phosphorylation and its translocation to the nucleus (50). The binding of IRF3 to IFN-stimulated response element (ISRE) in the promoters of type I IFN genes is thought to activate the transcription of these genes (59). However, non-structural proteins (NS1 and NS2) of RSV have been demonstrated to inhibit the induction of IFN-α/β in A549 cells and human macrophages (60,61). It was proposed that RSV NS1 protein limits IRF3 nuclear translocation through blocking its phosphorylation by decreasing the levels of 2 kinases upstream of IRF3 activation, TRAF3 and IKKε (62). RSV NS2 protein was also proposed to act on downstream molecules such as TRAF3 and ultimately inhibit IFN-β expression. Our results found that RSV infection indeed inhibits early IRF3 activation at 24 h pi, which is consistent with Hosakote's study (63). Moreover, oxidant $H_2O_2$ pretreatment further inhibits the early activation of IRF3 (Fig. 3F), indicating that an RSV-mediated redox-sensitive pathway probably inhibits IRF3 activation. Further studies can be done to investigate the mechanisms of interaction between NF-κB and IRF under conditions of RSV infection.

This study was conducted using A549 cells derived from human lung cancer tissue, which is similar to previous studies (41,56,64-66). A549 cells have also been widely used in the research of oxidative stress by RSV infection (41,64,67) or the alteration of TLR3 expression by RSV (21,68). Therefore, we believe that A549 cells, as an adenocarcinomic alveolar basal epithelial cell line, will have no intervening effect on the analysis and conclusion of the study. However, it will be interesting to investigate whether $H_2O_2$ and NAC have the same effects on RSV-induced TLR3, NF-κB and IRF3 expression in non-carcinomic primary epithelial cells in the future.

For production of type I IFN, RSV is generally considered as a poor inducer of IFN-α and IFN-β, comparing with other RNA viruses (69,70). However, RSV is previously reported to induce high levels of IFN-β expression in cultures of various types of human fibroblasts, respiratory epithelial cells, and mesenchymal stem cells (MSCs) (71-73). Besides, RSV is also reported to induce high levels of IFN-α expression in different subsets of dendritic cells (DC) (74-77). Furthermore, RSV treatment is also reported to induce a type I IFN response in both human cord blood-derived mast cell (CBMCs) and peripheral blood derived mast cells (78,79). Therefore, the results on the type I IFN production in responding to RSV infection are controversial and it seems that many different factors can determine the type I IFN production under the infection of RSV, such as different cell types and different viral strains (71,72,74-77,80). On these basis, we tentatively thought that the response of IFN-α and IFN-β to the infection of RSV long strain in A549 cells, which was related to the experiments in this study, might have various possible results. Thus, for the reason that the inducement of type I IFN in A549 cells may not be representative, we consider that it is not necessary to detect IFN-α and IFN-β in this study.

Taken together, we investigated the relationship between TLR3 expression and oxidative stress modulation in RSV-infected A549 cells in this study. We used oxidative stress agonist $H_2O_2$ and inhibitor NAC (equivalent to oxidant and antioxidant) to interfere with RSV infection from both positive and negative sides to determine the effect of oxidative stress on TLR3 expression and TLR3-mediated inflammatory and immune pathways. Our results showed that, in RSV-infected A549 cells, the production of hydroxyl free radical (·OH) and nitric oxide (NO) were induced, while the superoxide dismutase (SOD) activity was reduced. On the variation of gene expression, our results showed that both of mRNA and protein expression levels of TLR3 and NF-κB were up-regulated. Pretreatment of $H_2O_2$ plus RSV infection enhanced RSV-induced TLR3 and NF-κB expression, whereas Pretreatment of NAC plus RSV infection reduced them. These results indicated that oxidative stress was a critical regulator of TLR3 activation in RSV infection and suggested that oxidative stress might potentiate increasing the TLR3 expression in A549 cells after RSV infection, which might partly explain the enhancement of the associated downstream signaling pathway,

including NF-κB activity. The elevated TLR3 and NF-κB activities might be key factors in interpreting oxidative stress effects induction. These findings may contribute to the study of RSV pathogenesis and the development of RSV prevention and control.

To further confirm the role of oxidative stress discovered in this study, we plan to knock-out TLR3 and p-NF-κB genes using the CRISPR/Cas9 technique in RSV future work. First, we will synthesize three high-grade small-guide RNAs (sgRNAs) that could specifically identify TLR3 and p-NF-κB genes and inserted them into lenti CRISPRv2 plasmid. Second, 293T cells will be transfected with the recombinant sgRNA-lenti CRISPRv2 plasmid to yield subsequent sgRNA-Cas9 lentivirus prior to its further infection of A549 cells. Third, positive A549 cells will be screened using puromycin and be validated by PCR and western blot. Last, sequencing analysis will be adopted to confirm the mutation site of the obtained genes-knockout A549 cells.

Besides, we also plan to over-express TLR3 and p-NF-κB genes by lentiviral transfection to verify the function of oxidative stress. First, we will amplify TLR3 and p-NF-κB genes by RT-PCR and insert them into the lentiviral vector pLENTI-cGFP using the homologous recombination method. Second, the constructed recombinant vector will be confirmed by DNA sequencing and be co-transfected with psPAX2 and pMD2. G helper plasmids into HEK293T packaging cells to produce the lentiviral particles on the ratio of 3:2:1 by PEI transfection reagent. Third, the lentiviral particles will be transduced into A549 cells and the infection efficiency will be measured by Fluorescence Microscopy. Last, the GFP-tag-expressed cells will be sorted by the Flow Cytometer (FCM) after 2 weeks and the protein expressions of TLR3 and p-NF-κB and GFP in the stable cell lines will be confirmed by western blot.

Once the TLR3 and p-NF-κB gene deletion and over-expression are achieved, the established cell lines will be subjected to molecular biological tests in the cases of RSV infection and $H_2O_2$ or NAC intervention. We hope that these experiments will facilitate further investigation of the molecular mechanism of oxidative stress modulation of the TLR3 expression in RSV infection.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

MMW and SHH conceived and planned the study. XW performed the statistical analysis and JXC analyzed the data. WWL purified the RSV Long strain, and TS and HQ performed RT-PCR and western blot experiments. MMW, ML and CLZ carried out the chemical detection experiments. MMW, ML and CLZ wrote the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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