Antiviral immune mechanism of Toll-like receptor 4-mediated human alveolar epithelial cells type II

DANDAN WANG and JIE WANG

Department of Infectious Disease, Xuzhou Children's Hospital Affiliated to Xuzhou Medical University, Xuzhou, Jiangsu 221006, P.R. China

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Abstract. Expression of Toll-like receptor (TLR)4 and its downstream substances, myeloid differentiation factor 88 (MyD88), NF-κB p65, tumor necrosis factor-α (TNF-α) and GR in human alveolar epithelial cells type II (AEC II) infected with respiratory syncytial virus (RSV) were investigated, and the antiviral immune mechanism mediated by TLR4 was explored. Human AEC II were divided into $TLR4^{-/-}$ group, normal group and $TLR4^{+}$ group, and also into control group, RSV group and RSV+MP (methylprednisolone) group. MTT assay was used to measure the survival of cells after TLR4 knockout and overexpression, and the survival of normal cells after treatment with MP. The concentration of TLR4, MyD88, NF-κB p65, TNF-α, and GR was measured by ELISA after TLR4 knockout and overexpression. Reverse transcription-quantitative PCR (RT-qPCR) was used to measure the mRNA expression of the gene knockout and overexpression groups. RT-qPCR and western blot analysis were used to determine the expression of TLR4, MyD88, NF-κB p65 and GR in RSV and RSV+MP groups. The concentration of the detected substances in the $TLR4^{-/-}$ group was significantly lower than that in the normal group ($P<0.01$ and <0.001), and in the $TLR4^{+}$ group was significantly higher than that in the normal group ($P<0.05$, <0.01 and <0.001); the expression of RSV in the $TLR4^{-/-}$ group was significantly higher than that in the normal group ($P<0.001$), and in the $TLR4^{+}$ group was significantly lower than that in the normal group ($P<0.05$). The expression levels of TLR4, MyD88 and NF-κB p65 in the RSV and RSV+MP groups were significantly higher than those in the control group ($P<0.05$, <0.01 and <0.001), and the increase presented in the RSV+MP group was significantly lower than that in the RSV group ($P<0.05$ and <0.01). TLR4-mediated antiviral immunity of human AEC II can reduce the levels of TLR4, MyD88, NF-κB p65 and TNF-α and increase the level of GR, participating in the immune defense and reducing the damage of the viral epithelial cells of human type II alveoli, thus improving human immunity.

Correspondence to: Dr Jie Wang, Department of Infectious Disease, Xuzhou Children's Hospital Affiliated to Xuzhou Medical University, 18 Sudi Bei Road, Xuzhou, Jiangsu 221006, P.R. China
E-mail: unp1no@163.com

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Introduction

Alveolar epithelial cells type II (AEC II), also known as granule alveolar cells, are generally located in depressions on the alveolar surface, alveolar and alveolar junctions, and alveolar angles. Some studies have revealed that AEC II are the main precursor cells for alveolar damage repair during infection and tissue damage, and have strong immune function (1,2). AEC II are involved in the immune response through various ways (3), i.e., secreting a variety of antibiotics, such as lysozyme, expressing a variety of receptors, such as Toll-like receptors (TLRs) (4) and receptors for advanced glycation endproducts, and secreting pulmonary surfactant proteins (5,6). However, the specific mechanism by which AEC II generates immunity through these methods is not fully understood. Therefore, an antiviral immunity study of AEC II would be beneficial for both the basic medicine and clinical treatment.

TLRs belong to the family of pattern recognition receptors (PRRs). At present, 11 TLRs [TLR1-TLR10 (7) and TLR14] have been identified in the human genome. TLRs can be stimulated by microorganisms, recruiting specific adaptor proteins, and activating a series of signal cascades that trigger the body's immune response. As a type of PRRs, TLRs can recognize lipids, peptides and carbohydrates expressed by various pathogenic microorganisms (8-11). According to previous studies, there are two major signal transduction pathways in the cascade amplification reaction; one is the myeloid differentiation factor 88 (MyD88)-dependent pathway (12,13), and the other is the MyD88-independent pathway (14). The MyD88-dependent pathway is a transduction pathway in which all TLRs are involved, apart from TLR3. TLR4 is widely distributed in the immune cells, other than B cells, T cells, and NK cells. Cyr *et al* (15) have shown that pneumonia immune response to respiratory syncytial virus (RSV) infection is dependent on the complete TLR4 or MyD88 signaling pathway. Zhou *et al* (16) have indicated that TLR4 could mediate the production

of cellular inflammatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin-1β, and could be involved in the transduction of inflammatory signals and responses. Yan (17), and Akira and Takeda (18) have demonstrated that the MyD88 signaling pathway could activate the NF-κB signaling pathway, which could activate NF-κB and IFN-regulated factor 3 (IRF3). The activity of NF-κB is enhanced in viral myocarditis. The use of NF-κB blocker can effectively block the condition of myocarditis and inhibit the expression of inflammatory cytokine TNF-α in cardiomyocytes (19). The aforementioned studies suggest that TLR4 may be involved in the body's immune response and may be regulated by the TLR4/MyD88/NF-κB signaling pathway.

RSV is a single-stranded negative RNA virus that belongs to pneumoviruses, and is the most common pathogen of lower respiratory tract infections in infants and young children. Of all the TLRs found in the human body, TLR3, TLR4 and TLR7 are the ones most closely related to RSV (20). TLR4 is mainly expressed on the surface of cell membranes. When RSV invades, TLR4 can recognize the F protein of RSV and induce the excessive activation of MyD88 and NF-κB in the downstream, leading to the production of cellular inflammatory factors (TNF-α and IL-2) (21), thus causing diseases, such as pneumonia and asthma. However, it is not entirely clear which signal factors are involved in the transduction and expression level downstream of the immune process. Studies have shown that glucocorticoids (GCs) can act on innate immune cells (22) and protect the body from the damage of lipopolysaccharides (23). Therefore, it is not clear whether TLR4 induces an immune response, and whether glucocorticoid receptor (GR) is involved, in the process of antiviral immunity is not yet fully understood.

In the present study, RSV-infected human AEC II A549 were used, and RSV-infected GR agonist methylprednisolone (MP) was utilized to pre-intervene human AEC II A549 and establish an *in vitro* cell model, in order to evaluate the effect of RSV on the proliferation of A549 cells by MTT. ELISA, reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis were carried out to investigate the effect of MP pre-intervention on the expression of TLR4, MyD88, NF-κB p65, TNF-α and GR. The present study aimed to investigate the effect of MP on the expression of cytokines in lung epithelial cells infected with RSV and explore the TLR4-mediated immune mechanism in human AEC II during antiviral immunity.

Materials and methods

Virus and cells. Respiratory virus (international standard strain long) was purchased from Wuhan Procell Life Science Co., Ltd., human AEC II A549 were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and both gene knocked out and overexpressed TLR4 human AEC II A549 cells were purchased from Wuhan Servicebio Technology Co., Ltd. The study was approved by the Ethics Committee of Xuzhou Children's Hospital Affiliated to Xuzhou Medical University (Xuzhou, China).

Drugs and main reagents. Fetal bovine serum and DMEM (both from Gibco; Thermo Fisher Scientific, Inc.); MTT kit (cat. no. 11465007001; Roche Diagnostics); MP (cat. no. BP249; Sigma-Aldrich; Merck KGaA); TRIzol® total RNA extraction kit (cat. no. T9424; Sigma-Aldrich; Merck KGaA). Reagents for RT-qPCR experiments, such as Taq DNA polymerase, were purchased from Wuhan Servicebio Technology Co., Ltd. Human TLR4 (cat. no. E0753h), MyD88 (cat. no. E1707h), NF-κB p65 (cat. no. E1824h), TNF-α (cat. no. E0133h) and ELISA kits were purchased from Wuhan EIAab Science Co, Ltd. Murine NF-κB subunit p65 monoclonal antibody (cat. no. 13752-1; Cayman Chemical Company) and murine TLR4 antibody (cat. no. 3251-100) (both from BioVision, Inc.); murine MyD88 antibody (cat. no. ant-464; ProSpec); GR antibody (cat. no. BS6617; Bioworld Technology, Inc.); HRP-labeled goat anti-mouse IgG secondary antibody (cat. no. SE131; Solarbio Science & Technology Co., Ltd.).

Main instruments. Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.); StepOne™ fluorescence Real-Time PCR (Applied Biosystems; Thermo Fisher Scientific, Inc.); Mini Trans-Blot Cell protein transfer instrument, electrophoresis instrument, and VersaDoc Mode 5000 gel imaging analysis system (all from Bio-Rad Laboratories, Inc.).

Determination of viral infection. The viral solution was serially diluted 10 times in a centrifuge tube from 10^{-1}-10^{-10} of the original solution. The diluted virus was inoculated into a 96-well plate, and 6 wells were set at each dilution, and 100 μl were inoculated per well. Cell suspension was added to each well for a final concentration of 2-3x10^5 cells/ml. The normal cell group (100 μl growth solution + 100 μl cell suspension) was set, the number of cytopathic effects was observed for 6 consecutive days, and the experimental results were recorded. The median tissue culture infective dose ($TCID_{50}$) of the virus was calculated according to the Reed-Muench method (24). $TCID_{50}$ was the concentration of the RSV used in the subsequent experiments.

Cell culture and grouping. Human AEC II A549 were cultured in a 25-cm^2 cell culture flask after trypsin digestion. After 24 h, the cell adherent growth state was observed. Human AEC II A549 were collected, digested by trypsin and inoculated in a 25-cm^2 cell culture bottle. The adherent growth status of cells was observed after 24 h. The study was divided into two parts: a) The knocked out and overexpressed TLR4, as well as the normal expressed A549 cells were divided into three groups: i) $TLR4^{-/-}$ group, ii) normal group, iii) $TLR4^{+}$ group. All three groups were cultured with DMEM containing 100 μl RSV for 24 h. b) The normally expressed A549 cells were divided into three groups: i) Control group: A549 cells cultured in DMEM; ii) RSV group: A549 cells cultured in DMEM containing 100 μl RSV for 24 h; and iii) RSV+MP group: A549 cells cultured for 4 h in DMEM containing 600 ng/ml MP, and then cultured in DMEM containing 600 ng/ml MP + 100 μl RSV for 24 h.

Cell viability determination by MTT asssay. A549 cells of the $TLR4^{-/-}$ group, normal group and $TLR4^{+}$ group were trypsinized and inoculated into 96-well plates, and then cultured for 24 h. RSV (100 μl) was added to each group of cells, and 6 duplicate wells were set as the drug group. At the same

Table I. qPCR primers.

Target	Forward primers	Reverse primers
β-actin	5'-GGAGCGAGATCCCTCCAAAAT-3'	5'-GGCTGTTGTCATACTTCTCATGG-3'
TLR4	5'-AGACCTGTCCCTGAACCCTAT-3'	5'-CGATGGACTTCTAAACCAGCCA-3'
MyD88	5'-GGCTGCTCTCAACATGCGA-3'	5'-CTGTGTCCGCACGTTCAAGA-3'
NF-κB p65	5'-ATGTGGAGATCATTGAGCAGC-3'	5'-CCTGGTCCTGTGTAGCCATT-3'
RSV	5'-ACCGGCTGTCTCGTATGAAT-3'	5'-CTGCCAACATCCGATCAGTG-3'
GR	5'-CCAAGCTTCGATTCAGCAGGCCACTACA-3'	5'-GGGGTACCTCACTTTTGAAACAGATTTTTG-3'

TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; RSV, respiratory syncytial virus; GR, glucocorticoid receptor.

time, the control group was set with the addition of 100 μl of DMEM. After cell incubation for 24 h in a CO_2 incubator, 10 μl of MTT (5 mg/ml) were added to each well, and the cells were cultured for 4 h with the addition of 150 μl dimethyl sulfoxide (DMSO) to each well. A microplate reader was used to measure the absorbance value (A value) at 570 nm. The cell survival rate was calculated as: Survival rate (%) = (A value of drug group)/(A value of control group) x100%.

A549 cells with normal TLR4 expression were digested with trypsin and inoculated in 96-well plates, and then cultured for 24 h. MP of different concentrations (0, 100, 200, 300, 400, 500, 600, 700 and 800 ng/ml) was added to each well for incubation for 4 h, and 100 μl RSV were added to the A549 cells that had been incubated by MP. Six complex wells were set in each well as the drug group, and the control group was set at the same time. A549 cells were incubated in a CO_2 incubator of constant temperature for 24 h and then 10 μl MTT (5 mg/ml) were added to each well. A total of 150 ml DMSO were added to each well after further culture for 4 h. The A value was determined at 570 nm using ELISA. The cell survival rate was calculated as: Survival rate (%) = (A value of drug group)/(A value of control group) x100%.

ELISA. A549 cells in the $TLR4^{-/-}$ group, normal group, and $TLR4^{+}$ group were trypsinized and then inoculated into 24-well plates for 24 h. The cells were digested with trypsin, and then collected with centrifugation at 25˚C for 4 min at 1,000 x g. The collected cells were washed 3 times with cold PBS, and were disrupted by ultrasonic wave. Cells were frozen and thawed 3 times repeatedly to break the cells as much as possible. The disrupted cells were centrifuged at 4˚C, at 1,500 x g for 10 min, and the supernatant was collected for later use. For each test substance, the experimental procedures were carried out according to the manufacturer's instructions of the specific ELISA kit. The optical density value of each well at 450 nm was measured using a microplate reader. A standard curve was drawn to calculate the concentration value of each substance according to the curve equation.

RT-qPCR. Total RNA was extracted from A549 cells of the $TLR4^{-/-}$ group, normal group, $TLR4^{+}$ group, control group, RSV group and RSV+MP group using TRIzol® reagent, and the absorbance value at 260 and 280 nm was measured by a micro-spectrophotometer for the content and purity determination of RNA. After incubating at 37˚C for 50 min and heating at 70˚C for 15 min, total RNA was reverse transcribed into cDNA, and PCR amplification was carried out. PCR reaction conditions were 95˚C for 30 sec, 95˚C for 15 sec, 60˚C for 30 sec, with a total of 40 cycles. β-actin was used as the internal reference. Three replicate wells were set in each group, and the relative expression of each gene was calculated by $2^{-\Delta\Delta Cq}$ (25). The primer sequences of β-actin, TLR4, MyD88, NF-κB p65, RSV and GR are presented in Table I.

Western blot analysis. A549 cells in control group, RSV group and RSV+MP group were digested with trypsin and inoculated into 25-cm^2 cell culture flasks according to the requirements of each group. Total protein was extracted from A549 cells using RIPA protein extraction buffer (G2002; Wuhan Servicebio Technology Co., Ltd.). RIPA lysate and protease inhibitor were prepared at 100:1 dilution). The total protein concentration in the supernatant was determined by BCA protein assay. A solution containing 70 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%), and the separated protein was transferred to a polyvinylidene fluoride membrane with the protein transfer instrument and blocked with 5% skim milk for 2 h at 25˚C. After washing, a murine TLR4 antibody diluted at 1:1,200, or a murine MyD88 antibody, or a murine NF-κB subunit p65 antibody was added and incubated overnight at 4˚C. After washing again, HRP-labeled rabbit anti-mouse IgG secondary antibody diluted at 1:2,000 was added and incubated at 37˚C for 2 h on a shaker. The reference protein GAPDH (1:1,000, 60004-1-Ig) was provided by Proteintech Group, Inc. Protein bands were visualized with electrochemiluminescence reagent (G2020-1; Wuhan Servicebio Technology Co., Ltd.) and the image strips were scanned with gray scale using VersaDoc Mode 5000 gel imaging analysis system. The relative level of each target protein was quantified by the gray value ratio of target protein/GAPDH using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used for the statistical analysis of the data. The experimental data were expressed as the mean ± standard error of the mean (mean ± SEM). One-way ANOVA was used to compare multiple groups of data and SNK-q was the post hoc test used. $P<0.05$ was considered to indicate a statistically significant difference.

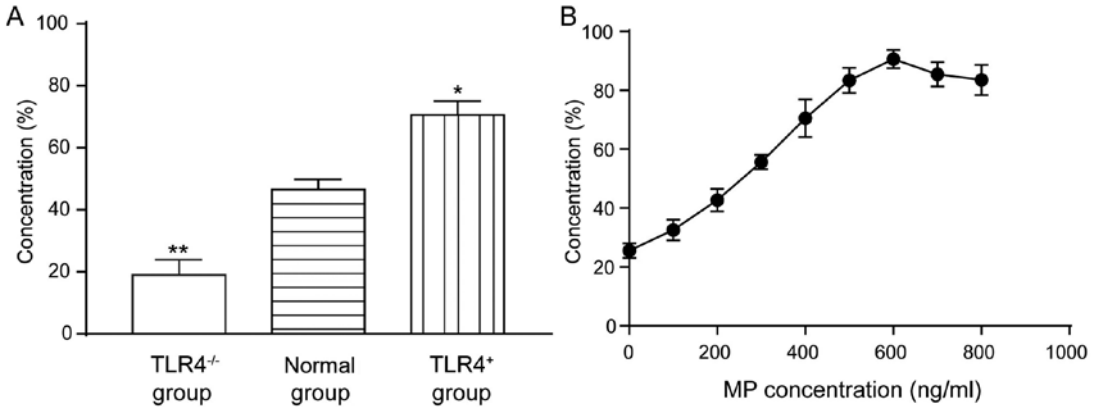

Figure 1. Effect of (A) TLR4 expression and (B) MP on the proliferation of A549 cells. *P<0.05, **P<0.01 vs. normal group. TLR4, Toll-like receptor 4; MP, methylprednisolone.

Results

Titration results of the median infective dose of RSV. Reed-Muench method was used to calculate the distance ratio as: [(infection percentage >50% - 50%)/(infection percentage >50% - infection percentage <50%)] xlog dilution, and then the following formula was used: log($TCID_{50}$) = dilution of infection percentage >50% + distance ratio. The experimental results showed that $TCID_{50}$ was $10^{-6.5}$/0.1 ml.

Effect of RSV on proliferation of human AEC II. In the MTT assay of TLR4 knocked out and overexpressed A549 cells, the cell viability of the $TLR4^{-/-}$ group was 19.58±3.54%, which was significantly lower than that of the normal group (47.86±2.58%) (P<0.01). The survival rate of cells in the $TLR4^{+}$ group (71.25±3.86%) was significantly higher than that in the normal group (P<0.05) (Fig. 1A), indicating that the expression of TLR4 can enhance the survival of cells after infection with the virus.

MP has a proliferation-promoting effect on RSV-incubated A549 cells, in the normal expression of TLR4 cells (Fig. 1B). In 100 μl of incubated cells, when the MP concentration was 0, 100, 200, 300, 400, 500, 600, 700 and 800 ng/ml, the promotion rate was 25.53±2.53, 32.56±3.48, 42.67±3.86, 55.62±2.40, 70.56±6.40, 83.35±4.25, 90.61±3.13, 85.45±4.21 and 83.52±5.21%, respectively, indicating that MP can play an antiviral role.

ELISA detection. The mass concentration of TLR4 in the $TLR4^{-/-}$ group was 0.0568±0.0153, in the normal group was 0.853±0.034, and in the $TLR4^{+}$ group was 2.4369±0.0541. Compared with the normal group, TLR4 mass concentration in the $TLR4^{-/-}$ group was significantly decreased (P<0.001), whereas in the $TLR4^{+}$ group was significantly increased (P<0.001) (Fig. 2A). The mass concentration of MyD88 in the $TLR4^{-/-}$ group was 0.5835±0.204, in the normal group was 2.326±0.113, and in the $TLR4^{+}$ group was 4.056±0.159. Compared with the normal group, the mass concentration of MyD88 in the $TLR4^{-/-}$ group was significantly decreased (P<0.01), whereas in the $TLR4^{+}$ group was significantly increased (P<0.01) (Fig. 2B). The mass concentration of NF-κB p65 in the $TLR4^{-/-}$ group was 0.732±0.0864, in the normal group was 2.552±0.095, and in the $TLR4^{+}$ group was 3.892±0.105. Compared with the normal group, the mass concentration of NF-κB p65 in the $TLR4^{-/-}$ group was significantly decreased (P<0.01), whereas in the $TLR4^{+}$ group was significantly increased (P<0.01) (Fig. 2C). The mass concentration of TNF-α in the $TLR4^{-/-}$ group was 7.206±0.586, in the normal group was 18.308±0.534, and in the $TLR4^{+}$ group was 30.582±0.489. Compared with the normal group, the mass concentration of the TNF-α in the $TLR4^{-/-}$ group was significantly decreased (P<0.01), whereas in the $TLR4^{+}$ group was significantly increased (P<0.01) (Fig. 2D). The mass concentration of GR in the $TLR4^{-/-}$ group was 30.892±2.62, in the normal group was 61.389±1.351, and in the $TLR4^{+}$ group was 69.25±2.158. Compared with the normal group, the mass concentration of GR in the $TLR4^{-/-}$ group was significantly decreased (P<0.01), whereas in the $TLR4^{+}$ group was significant increased (P<0.05) (Fig. 2E). The results revealed that the expression of TLR4 could promote the expression of its downstream regulatory factors MyD88, NF-κB p65, TNF-α and GR.

Gene expression of TLR4, MyD88, NF-κB p65, GR and mRNA expression of RSV in human AEC II. The relative expression of RSV in $TLR4^{-/-}$ group was 2.729±0.159, which was significantly higher than that in the normal group (P<0.001). The relative expression of RSV in the $TLR4^{+}$ group was 0.348±0.345, which was lower than that in the normal group, with a statistically significant difference (P<0.05) (Fig. 3). The results indicated that the expression of TLR4 could inhibit the replication of RSV in A549 cells.

The gene expression of TLR4 in the RSV group was 5.234±0.104 and in the RSV+MP group was 2.935±0.312, both of which were increased to different degrees compared with the TLR4 expression in the control group, presenting statistically significant differences (P<0.001 and <0.01, respectively). The degree of increase in the RSV+MP group was lower than that in the RSV group, with a statistically significant difference (P<0.01) (Fig. 4A). The gene expression of MyD88 in the RSV group was 3.123±0.241 and in the RSV+MP group was 1.678±0.192, both of which were increased to different degrees compared with the MyD88 expression in the control group, presenting statistically

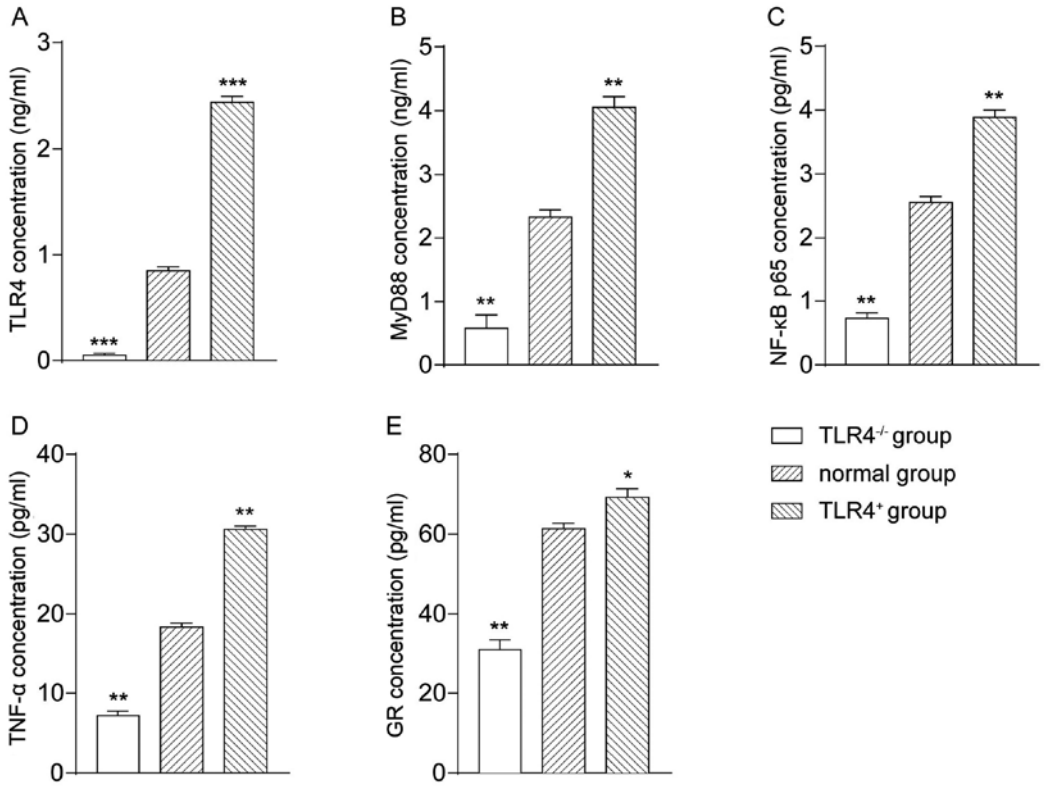

Figure 2. Determination of mass concentration of TLR4, MyD88, NF-κB p65, TNF-α, and GR in each group of A549 cells via ELISA. Mass concentration of (A) TLR4, (B) MyD88, (C) NF-κB p65, (D) TNF-α, and (E) GR in each group of cells. *P<0.05, **P<0.01, ***P<0.001 vs. normal group. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; TNF-α, tumor necrosis factor-α; GR, glucocorticoid receptor.

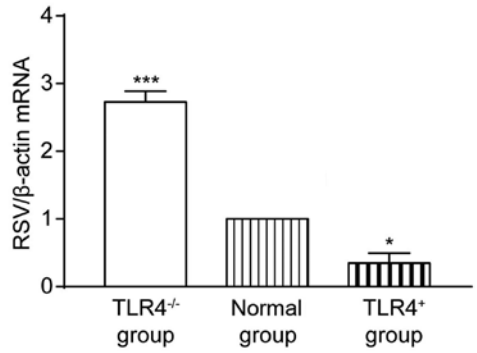

Figure 3. Determination of RSV mRNA expression in TLR4$^{-/-}$ group, normal group, and TLR4^{+} group using RT-qPCR. *P<0.05, ***P<0.001 vs. normal group. RSV, respiratory syncytial virus; TLR4, Toll-like receptor 4.

significant differences (P<0.001 and <0.05, respectively). The degree of increase in the RSV+MP group was lower than that in the RSV group, with a statistically significant difference (P<0.05) (Fig. 4B). The gene expression of NF-κB p65 in the RSV group was 2.951±0.235 and in the RSV+MP group was 1.791±0.157, both of which were increased to different degrees compared with the NF-κB p65 expression in the control group, presenting statistically significant differences (P<0.001 and <0.01, respectively). The degree of increase in the RSV+MP group was lower than that in the RSV group, with a statistically significant difference (P<0.05) (Fig. 4C). The gene expression of GR in the RSV group was 0.4811±0.086, which was significantly decreased compared with that in the control group (P<0.001), and in the RSV+MP group was 0.891±0.057, which was significantly increased compared with that in the RSV group (P<0.01) (Fig. 4D). The results showed that the infection with RSV could lead to increased expression of TLR4, MyD88 and NF-κB p65, and to a decreased expression of GR, whereas MP could reverse this phenomenon.

Protein expression of TLR4, MyD88, NF-κB p65 and GR in human AEC II. The protein expression of TLR4 in the control group was 0.161±0.026, in the RSV group was 0.568±0.048, and in the RSV+MP group was 0.332±0.037. The protein expression of MyD88 in the control group was 0.104±0.056, in the RSV group was 0.538±0.046, and in the RSV+MP group was 0.304±0.054. The protein expression of NF-κB p65 was 0.055±0.284 in the control group, 0.432±0.059 in the RSV group, and 0.097±0.013 in the RSV+MP group. The protein expression of GR in the control group was 0.868±0.125, in the RSV group was 0.147±0.052, and in the RSV+MP group was 0.488±0.086. The protein expression levels of TLR4, MyD88 and NF-κB p65 in the RSV and RSV+MP groups were significantly higher than those in the control group, with statistically significant differences (P<0.05 and <0.001). The increase in the RSV+MP group was lower than that in the RSV group, with statistically significant differences (P<0.05 and <0.01). The protein expression of GR in the RSV and RSV+MP groups was significantly lower than that in the control group (P<0.05 and <0.001). There was a significant difference in the expression of GR protein between the RSV+MP and RSV groups (P<0.05) (Fig. 5).

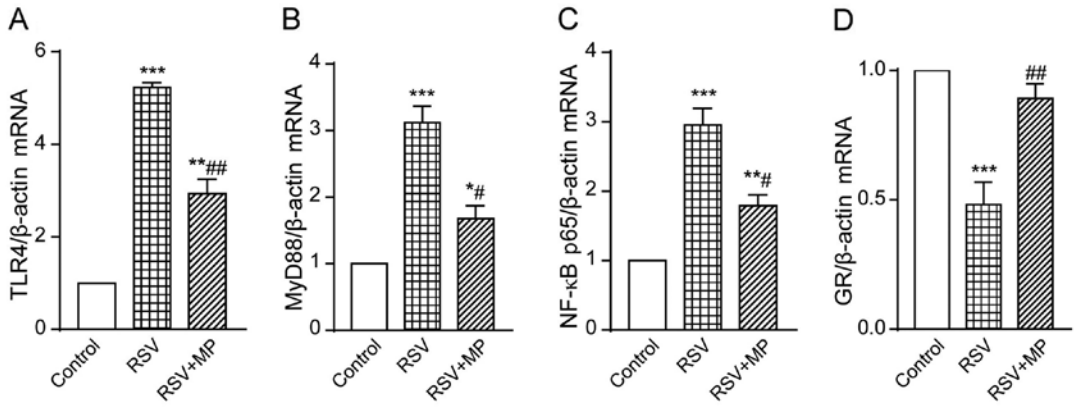

Figure 4. Determination of TLR4, MyD88, NF-κB p65, and GR gene expression in A549 cells of the control group, RSV group and RSV+MP group using RT-qPCR. Gene expression levels of (A) TLR4, (B) MyD88, (C) NF-κB, and (D) GR in each group. *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. RSV. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; GR, glucocorticoid receptor; RSV, respiratory syncytial virus; MP, methylprednisolone.

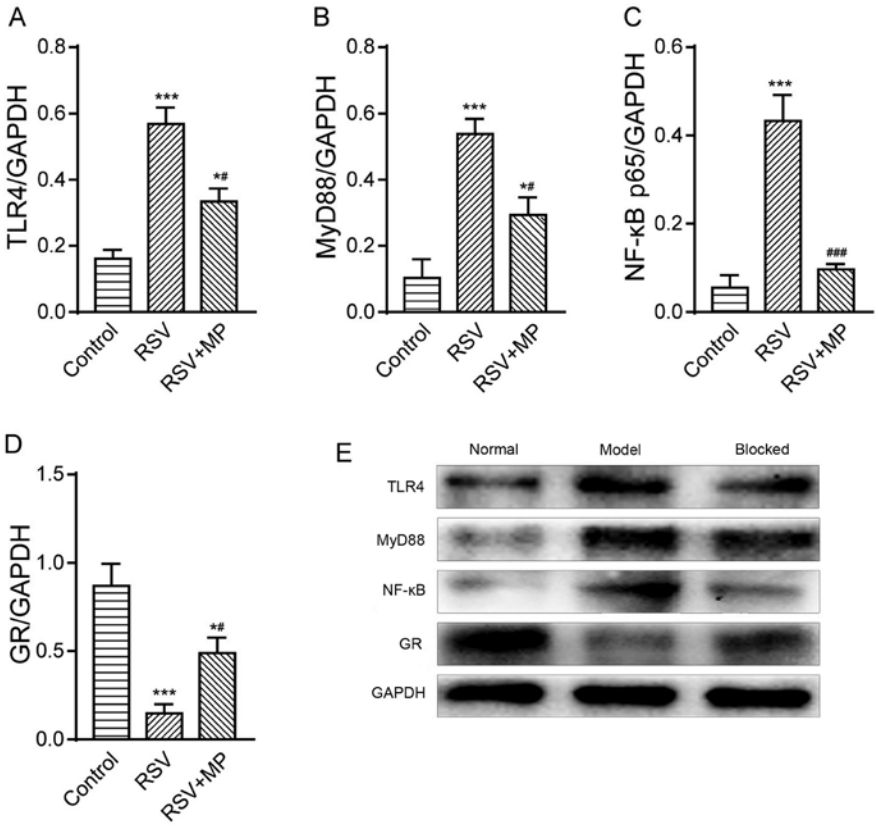

Figure 5. Determination of TLR4, MyD88, NF-κB p65 and GR protein expression in A549 cells of the control group, RSV group and RSV+MP group using western blot analysis. Protein expression levels of (A) TLR4, (B) MyD88, (C) NF-κB, and (D) GR in each group. (E) Western blots. *P<0.05 and ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.01 vs. RSV. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; GR, glucocorticoid receptor; RSV, respiratory syncytial virus; MP, methylprednisolone.

Discussion

Viral pneumonia is a common respiratory disease, and RSV is the most important pathogen of the viral lower respiratory tract infections in infants and young children. Delgado *et al* (26) have suggested that infants lack protection, probably due to low antibody affinity for protective epitopes, and low antibody affinity results in lack of TLR stimulation. Thus, activation of TLRs plays an important role in the immune process.

In the present study, the MTT assay results revealed that knockdown of the TLR4 gene had a certain inhibitory effect on the proliferation of A549 cells, whereas overexpression could improve proliferation. In the subsequent ELISA experiment, overexpression of TLR4 gene increased the concentration of TLR4, MyD88, NF-κB p65, TNF-α and GR, indicating that the expression of TLR4 could promote cell survival. RT-qPCR and western blot analysis results revealed that the levels of TLR4, MyD88 and NF-κB p65 in RSV and RSV+MP groups were

significantly higher than those in the control group; however, the increase in the RSV+MP group was significantly lower than that in the RSV group. In the RSV group, the level of GR was significantly lower than that in the control group; however, the level of GR in the RSV+MP group was not significantly different from that in the control group. After pretreatment with GR activator MP, RSV had a certain reversal effect on the inhibition of A549 cells, indicating that activation of GR could alleviate the damage of RSV on A549 cells. It was speculated that in RSV-infected AEC II cells, TLR4 might be the starting point for initiating antiviral immunity, regulating the immune response of the body by regulating TLR4/MyD88/NF-κB p65 signaling pathway.

GR is a member of the conserved nuclear receptor superfamily and a transcription factor which is present in the cytoplasm of various cells of the body, including α and β receptors. Among them, α receptor plays a major role (27). Under normal physiological conditions, GR forms a complex with other proteins, such as heat shock protein 90, preventing the GR receptor from entering the nucleus and interacting with DNA (28). However, when GR is combined with GC, the conformation of heat shock protein 90 changes, causing the GR receptor to separate from the complex and enter the nucleus, resulting in enhanced transcriptional activity of the target gene and production of corresponding proteins, such as adhesion molecules. GR can also block the transcription of related transcription factors and inhibits the production of related proteins, such as IL-1 and TNF-α. At the same time, the activation of GR can interact with NF-κB to inhibit the secretion of inflammatory factors, such as IL-1 and TNF-α in cells (29). A study by Kamiyama *et al* (30) has revealed that GR also plays an important regulatory part in the inflammatory response. GR can inhibit the activation of p38 MAPK and prevent the cascade of downstream inflammatory factors (31,32). GR can also destroy the downstream product of TLR4, the IRF3, thereby inhibiting the expression of related inflammatory genes. Therefore, GR was highly likely to play an important role in the immune response. In the present study, the experimental results on concentration and expression levels, suggest that insufficient or low activity of GR levels could cause a large number of release of inflammation factors, aggravating the human AEC II injury. However, in the use of preincubation of GR activator MP, the cell damage was relieved, suggesting that GR was involved in immune response and could reduce cell damage.

In the present study, the results revealed that RSV infection of human AEC II A549 pretreated with the GR activator MP could reduce the activation of TLR4 and downregulate the expression of downstream MyD88, NF-κB p65, and TNF-α, indicating that upregulation of GR expression is involved in antiviral protection. TLR4 and GR, as key parts of the overall pathway, can provide a theoretical basis for the clinical viral infections in the lungs.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DW and JW conceived and designed the study, collected, analyzed and interpreted the experimental data, drafted the manuscript, and revised it critically for important intellectual content. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Xuzhou Children's Hospital Affiliated to Xuzhou Medical University (Xuzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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