

Original Article

Toll-like receptor and its signaling pathway mediated immune escape in rats with minimal change nephritic syndromes induced by RSV

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Abstract: Objective: To prove that immune escape is involved in the pathogenesis of respiratory syncytial virus (RSV) by inhibiting Toll-like receptor (TLR) signaling pathway. Methods: We established a rat model with nephropathy induced by RSV for different days (4, 8, 14, 30, 60, 90, 120 d) to seek the evidence of RSV immune escape in kidney, lung and spleen tissues as well as PBMCs, meanwhile, the expressions of TLR3, TLR4 and NF- κ B, IFN- γ and IL-13, RSV fusion (RSV-F) protein as well as 24 h urine protein were compared and analyzed via real time fluorescent quantitative PCR detection, indirect immunofluorescent assay, ELISA and flow cytometry, to explore the correlations between persistent RSV infection and the immune response mediated by TLR and its signaling pathway. Results: RSV continuously survived in kidney, lung and spleen for 120 days. The expressions of TLR3, NF- κ B and IL-13 in each group changed with the RSV infection time: compared with the normal healthy rats, their expressions were significantly increased on day 4, 14, 90 and 120 after the virus inoculation when the expression of RSV was at high level, ($P < 0.05$); however, when RSV survived at a low-replication level, TLR3 was obviously inhibited ($P > 0.05$). Moreover, TLR4 and IFN- γ expressions had no significant difference compared with the control group ($P > 0.05$), except that TLR4 had a significant increase on day 4 and an apparent reduction on day 60, while IFN- γ elevation on Day 4 and 14. In addition, 24 h urine protein increased gradually and peaked at day 60. Conclusion: The continuous infection of RSV mainly suppressed the immune response via inhibiting the TLR4, TLR3 and IFN- γ signal pathway, indicating that the suppression of TLR4 and TLR3 signal pathways might be an essential mechanism of RSV immune escape, therefore RSV could continuously infect the body via immune escape, which is an important link of causing T-cell dysfunction and minimal change nephritic syndrome.

Keywords: Minimal change nephrotic syndrom, respiratory syncytial virus, TLR, signaling pathway, immune escape

Introduction

The pathogenesis of Minimal change nephritic syndrome (MCNS) and how it induces T-cell dysfunction haven't been well elucidated. In our previous studies, we performed long-term systematic researches and confirmed that respiratory syncytial virus (RSV) infection played an important role in the development of MCNS [1-3], and also found Toll-like receptor (TLR) might be involved in this process [4, 5]. Briefly, RSV was inoculated into rats and the changes of proteinuria and foot process were observed, finding that it was extremely similar to human MCNS, however, at the late stage of RSV inocu-

lation (30 days later), inflammation response induced by virus infection was alleviated while proteinuria and podocyte fusion were increased, indicating there exists other possible mechanisms [3].

Some reports suggested that there might exist RSV immune escape, for example, antibodies produced during RSV infection couldn't provide permanent protection, leading to repeated infection of RSV [6-10]. Moreover, F protein of RSV was proved to be capable of inhibiting the activation and proliferation of T cells, selectively interfering the differentiation of T cells, decreasing the expression of effector T cells

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and preventing the acquired immune response, thus delaying the virus scavenging [11]. So we wonder that if there also exists such kind of immune escape in rats with RSV nephropathy. If yes, what's the mechanism of RSV immune escape? How is RSV continuous infection inducing the development of nephropathy? Is that related to the inhibition of T cell proliferation? And what's the effect of the immune system of the body?

Recently, Toll-like receptor (TLR) and its signaling pathway has been a hot spot in the researches, which plays an essential role in pathogen identification and immune activation and participates in the pathogenesis of many diseases as well [12-14]. Additionally, mycobacterium tuberculosis can induce immune suppression by inducing TLR2-dependent mechanisms so as to promote IL-10 release, and inhibit host defense responses as well as increase the body's susceptibility, thus to guarantee its long-time survival in the cell [15]. The same survival mechanism is also seen in small intestine *Yersinia* bacteria, *Candida Albicans* etc. [16, 17]. Therefore, we hypothesized there might have RSV immune escape through the interference or inhibition of the TLR pathway, so as to participate in the pathogenesis of MCNS.

In this study, we established RSV rat model with nephropathy to specifically detect the expression of RSV in kidney, spleen and lung tissues to prove RSV immune escape, meanwhile, we analyzed the correlation between RSV persistent infection and the immune response characterized with TLR and its signal pathways as well to verify that immune escape is one important link in the pathogenesis of MCNS.

Materials and methods

Animal and experiment methods

Forty healthy male SD rats, aged 5-6 weeks with weight of 150-200 g, were provided by Huaxi Animal Center of Sichuan University, and all of them no special pathogen bacteria. Human RSV long strain and Hela cell strain were purchased from the Chinese Academy of Preventive Medicine. RSV fusion protein antibody was purchased from American Chemico Company. Rabbit anti-mouse TLR3, TLR4 antibody, sheep anti-mouse IFN- γ antibody, anti-rat

NF- κ B antibody were all purchased from American Santa Cruze Company; FITC labeled sheep anti-rabbit IgG was purchased from American Pierce Company.

Cell culture and bacterial infection

Hela cells were cultured in DMEM medium with 10% fetal bovine serum (Gibco, USA). RSV strain was inoculated into Hela cells in logarithmic growth phase and incubated in an incubator (SANYO) with 5% CO₂ under 37°C. When the obvious cell fusion was most obvious, forcedly shake off the cells from the wall of the bottle, then frozen and thaw for 3 times, and centrifugalize at 4°C (2000 g) for 10 min. Finally, collect the supernatant containing RSV and preserved at -70°C. Plaque forming assay was performed to titrate virus (the measured the plaque-forming unit (PFU)=6*10⁹/ml) before inoculating to rats.

Animal inoculation

Forty rats were reared in metabolic cages individually and randomly divided into 8 subgroups with 5 rats in each groups and inoculated with RSV suspension (6*10⁶ PFU) by intranasal drop (0.2 ml) and intraperitoneal injection (0.4 ml), once per day for continuous 3 days. The rats were sacrificed at 4, 8, 14, 30, 60, 90 and 120 d since the first RSV inoculation and named as RSV₄, RSV₈, RSV₁₄, RSV₃₀, RSV₆₀, RSV₉₀ and RSV₁₂₀, respectively. Other 5 normal rats without treatment were used as the control group, especially, they and other groups' rats were fed separately. The skin, behavior, appetite, breathing and urine appearance of each group were observed and recorded, and the weights when inoculating and before sacrifice were recorded.

Sample collecting

The urine sample was collected for 24 h from metabolic cages before sacrifice, and 5 ml was used for detection. The rats were anesthetized via intraperitoneal injection with 10% chloral hydrate, and then the chest and abdominal cavity were exposed under aseptic condition. 2 ml heart blood was collected for further measurement. After removing renal capsule and perirenal connective tissues, the kidneys were rinsed with normal saline, and renal cortex about the size of rice was quickly sheared and put in 1 ml Trizol for total RNA extraction. Another little

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Table 1. Sequence of primers and probes

Genes	Primers/Probes	Nucleotide Sequence 5' to 3'	bp
TLR3	Forward	ATTCATGCCCGAAAGACAG	215
	Reverse	ACCTTGAATCTTCTGCACAG	
	Probe	CTCCCAATGGAAGAACAAGAC	
TLR4	Forward	GAAGCTATAGCTTCACCAAT	134
	Reverse	GATAGGGTTTCCTGTGTCAGT	
	Probe	CACACCTGGATAAATCCAGCCAC	
IFN- γ	Forward	CACCTGATCACTAACTTCTT	103
	Reverse	CAGCTTTGTGCTGGATCTGT	
	Probe	CATCGCCAAGTTCGAGGTGAAC	
IL-13	Forward	CTGAGCAACATCACACAAG	169
	Reverse	GGTTACAGAGGCCATTCAAT	
	Probe	CACGCTCCATACCATGCTGC	
RSV-F	Forward	GAAGGAGAAGTGAACAAGATCAAA	325
	Reverse	CATTTGTTATACCGATATCATTGAT	
	Probe	CACACCAGCTGCCAACAACC	
β -actin	Forward	GCCAACACAGTGCTGTCT	114
	Reverse	AGGAGCAATGATCTTGATCTT	
	Probe	ATCTCCTTCTGCATCCTGTC	

piece of renal tissue was fixed with 3% glutaraldehyde in phosphate buffer solution for electron microscopy observation. The remaining kidney and lung tissues were fast frozen at -70°C for immune-fluorescence detection.

Real time fluorescent quantitative PCR detection

Primer design and total RNA extraction: The primers were designed as shown in **Table 1**, they all purchased from Shanghai Sangon Biotech Company. Total RNA extraction was performed as follows: 30-50 mg tissue was cut-up and put into a 1.5 ml EP tube containing 1 ml Trizol, then homogenized quickly. 0.2 ml chloroform was added, and the tube was closed and violently oscillate for 15 s. Then the sample was incubated at $15-30^{\circ}\text{C}$ for 2-3 minutes followed by centrifuging (12000 g) at 4°C for 15 min. The supernatant was mixed with 0.5 ml isopropyl alcohol to precipitate RNA, and then centrifuged (12000 g) at 4°C for 10 min. Discard the supernatant, and the RNA precipitation was wash with 1 ml 75% ethanol, and centrifuged at 7500 g for 5 min. Next, the supernatant was removed and precipitation was air-dried and dissolved in 50 μl ultrapure water without RNA enzyme.

Reverse transcription: The extracted total RNA was taken 5 μl as template to reversely tran-

script into cDNA according to the instruction of RevertAid (MBI) reverse transcription kit. The reaction system was as follows: 5 μl cDNA and 0.36 μl dNTP (25 mM), 3 μl MgCl_2 (25 mM), 15.34 μl ddH₂O, 3 μl 10 \times buffer (Mg^{2+} free), 1 μl upstream primers, 1 μl downstream primers, 1 μl TaqMan probe, and 0.3 μl Taq enzyme (PROMEGA) (5 U/ μl), and the final volume was 30 μl . β -actin was applied as internal reference.

R-T PCR: Then detection was performed on FTC2000 (FUNGLYN, Shanghai) quantitative PCR instrument, and parameters was set as 94°C for 2 min, 94°C 20 s, 50°C (RSV-F)/ 52°C (TLR3)/ 50°C (TLR4)/ 50°C (IFN- γ)/ 52°C (IL-13)/ 50°C (β -actin) for 30 s, 60°C for 40 s, and 45 cycles, 72°C for 5 min and detect fluorescence at 60°C . Then the gene expression levels of RSV-F, TLR3, TLR4, IFN- γ , IL-13 and β -actin were

obtained. When the fluorescence intensity of sample increased to certain threshold, the number of amplification cycles (Ct value) was in a linear relationship with the logarithm of initial template copy number, and $\Delta\text{Ct}=\text{Ct}(\text{sample})-\text{Ct}(\beta\text{-actin})$. The sample without cDNA was used as negative control.

Indirect immunofluorescent assay indirect immunofluorescent assay ELISA

Fresh kidney, lung and spleen tissues were quickly sliced in 4-5 μm section after frozen, and then the slices were transferred to polylysine coated film. After dried, they were fixed in cold acetone for 10 min and washed with 0.1 mol/L PBS for 5 min*3 times. To detect the expressions of TLR3 and NF- κB , 1% Triton X-100 (100 μl) were added, 5 min later, wash with 0.1 mol/L PBS for 5 min*3 times again. Then bovine serum was applied to block for 40 min before wash with 0.1 mol/L PBS for 5 min*3 times. Next, add primary antibody (1:100) and incubate in humidity chamber for 30 min at 37°C and overnight at 4°C . Next day, wash the slices with 0.1 mol/L PBS for 5 min*3 times and add FITC labeled secondary antibody (1:100) and incubate in dark surrounding for 1 h at 37°C followed by washing with 0.1 mol/L PBS for 5 min*3 times. Finally, 50% glycerol was used to seal the films before observing

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Table 2. Comparison of general conditions of rats inoculated with RSV with control group

Groups	Weight growth	Appetite	Fur gloss	Running nose	Breathing rate	Serous effusion
Control	Normal	Normal	Normal	None	Normal	None
RSV ₄	Slow	Decreased	Reduced	More common	Tachypnoea	Occational
RSV ₈	Slow	Decreased	Reduced	More common	Tachypnoea	A few
RSV ₁₄	Slow	Decreased	Reduced	More common	Tachypnoea	Occational
RSV ₃₀	Normal	Normal	Reduced	None	Normal	All
RSV ₆₀	Normal	Normal	Reduced	None	Normal	None
RSV ₉₀	Normal	Normal	Reduced	None	Normal	Occational
RSV ₁₂₀	Normal	Normal	Reduced	None	Normal	None

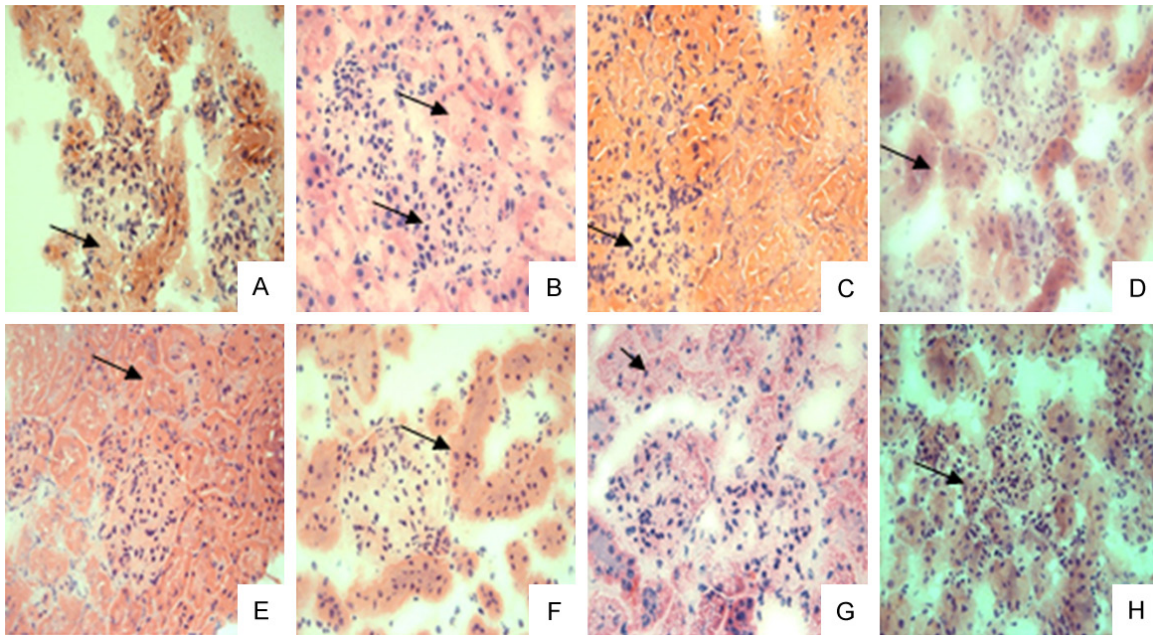


Figure 1. Histopathological finding in kidneys of RSV-inoculated rats via HE staining under light microscopy ($\times 400$). (A) RSV₄; (B) RSV₈; (C) RSV₁₄; (D) RSV₃₀; (E) RSV₆₀; (F) RSV₉₀; (G) RSV₁₂₀; (H) Control group. As arrows indicate, glomerular congestion and enlargement see in (A-C); renal tubular epithelial cell edema and expansion see in (B-G).

Table 3. The volume of proteinuria in 24 hours of each group ($\bar{x} \pm s$, mg/24 h)

Groups	n	Proteinuria (mg/24 h, $\bar{x} \pm s$)	P
Control	5	2.844 \pm 1.132	
RSV ₄	5	3.500 \pm 2.155	>0.05
RSV ₈	5	10.264 \pm 1.884	<0.05
RSV ₁₄	5	6.845 \pm 2.363	<0.05
RSV ₃₀	5	16.149 \pm 4.768	<0.05
RSV ₆₀	5	29.24 \pm 4.746	<0.05
RSV ₉₀	5	17.94 \pm 2.705	<0.05
RSV ₁₂₀	5	20.25 \pm 6.025	<0.05

under fluorescence microscope. Negative control slices used PBS instead of primary antibody.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) which were obtained from venous blood that dealt with EDTA anticoagulant of RSV infected rats. Cell surface was stained by phycoerythrin labeled anti-mouse TLR4 antibody (Abcam) for 30 min at 4°C. The detection was performed on FACS Aria machine and Cell Quest software (BD).

Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines in kidney, lung, spleen tissues homogenates as well as the serum of rats were detected via commercial Double Antibody Sandwich ELISA following the instructions on kits: IL-13

Table 4. Comparison of RSV-F mRNA expression in RSV-inoculated rats among different groups

Groups	ΔCt ($\bar{x} \pm s$)		
	Kidney (n=5)	Lung (n=5)	Spleen (n=5)
RSV ₄	9.125±2.287* [#]	12±2.150* [#]	8.5±2* [#]
RSV ₈	17.25±3.014	16.8±5.901	16.7±5.106
RSV ₁₄	7.7±3.290* [#]	10.3±2.928* [#]	8±2.208* [#]
RSV ₃₀	14.375±4.785	18.8±4.550	16.6±2.191
RSV ₆₀	11±5.280*	17.1±3.267*	13.25±1.190*
RSV ₉₀	6.8±0.837* [#]	11.4±0.652* [#]	11.7±3.818* [#]
RSV ₁₂₀	10.333±3.251*	10.917±1.625* [#]	8.917±1.530* [#]
Total	10.682±4.609	13.819±4.514 ^Δ	11.829±4.384*

*P<0.05, vs. RSV₈; [#]P<0.05, vs. RSV₃₀; ^ΔP<0.05, vs. kidney and spleen.

(Biosource); IFN-γ (Shenzheng JingMei Biotech Co., Ltd).

Statistical analysis

SPSS 10.0 software was adopted for statistical analysis. The measurement data were expressed as mean ± standard deviation, and the comparisons between groups were examined by variance analysis or t test. P<0.05 were considered as significant difference.

Result

General information

The weight of rats in RSV inoculation groups was lower than that of the control group, and the RSV infected rats showed different degrees of lethargy, decreased appetite, decreased activity and reduced fur gloss. During the early stage (Day 4-14), rats in RSV inoculation groups had running nose, nasal mucosa ulceration, tachypnea and sneezing, however, at the late stage (after Day 30), respiratory symptoms were not obvious and the appetite was improved. Some of the RSV inoculated rats showed peritoneal and (or) pleural effusion (Table 2).

Pathological analysis of kidney tissue

Enlarged kidneys could be observed in some rats with naked eyes. HE staining was indicative of glomerular congestion or swelling in the rats of RSV₄, RSV₈ and RSV₁₄ groups; renal tubular was observed with epithelial cell cloudy swelling, vacuolar degeneration and tubular

cavity expansion with some parts showing transparent pink, which was obvious on day 4 (RSV₄) and day 8 (RSV₈) but alleviated after 30 days (RSV₃₀, RSV₆₀, RSV₉₀ and RSV₁₂₀). Under electron microscope, the glomerular structure in RSV₄ group and control group were all complete and continuous without basement membrane thickening, while in RSV₈ group, partial glomerular endothelial cells appeared swelling, nuclear chromatin clumped, mitochondrial swelling. And from day 14, all the RSV showed different degrees of podocyte fusion, which was more obvious in RSV₆₀, RSV₉₀ and RSV₁₂₀ groups with visible glomerular podocyte segmental fusion and remarkable endothelial cell hyperplasia.

Whereas in RSV₉₀ group, podocyte fusion of partial glomerulus was not clear and the hyperplasia of the mesangial cells was found in RSV₁₂₀ group (Figure 1).

Quantification of 24 h urinary protein in inoculated rats

After RSV inoculation, the 24 h urine protein increased gradually and peaked at day 60: RSV₆₀>RSV₁₂₀>RSV₉₀>RSV₃₀>RSV₈>RSV₁₄>RSV₄>control group. There was no significant difference between control group and RSV_{4,14} groups (Table 3).

Detection of RSV-F mRNA and protein

The mRNA expression of RSV-F in kidney, lung and spleen tissues of nephropathy rats induced by RSV was detected by Real-time Quantitative PT-PCR and immunofluorescent assay respectively. RSV mRNA expression was at high level on day 4, but decreased a little on day 8, while enhanced on day 14 and then declined and reached the lowest level at day 30, however, increased again on day 90 following with down-trend until day 120. Additionally, its protein expressions in tissues of kidney and spleen were higher than that in lung tissue (Table 4). Meanwhile, RSV-F protein expression was also observed in kidney, lung and spleen tissues of RSV infected rats after immunofluorescent (FITC) staining, and the expression was higher on glomerulus than on renal tubular (Figure 2), which was also expressed in lung bronchiole and epithelial cells. Furthermore, in spleen tissues, the RSV-F protein was expressed in a focal and scattered way, commonly seen in the

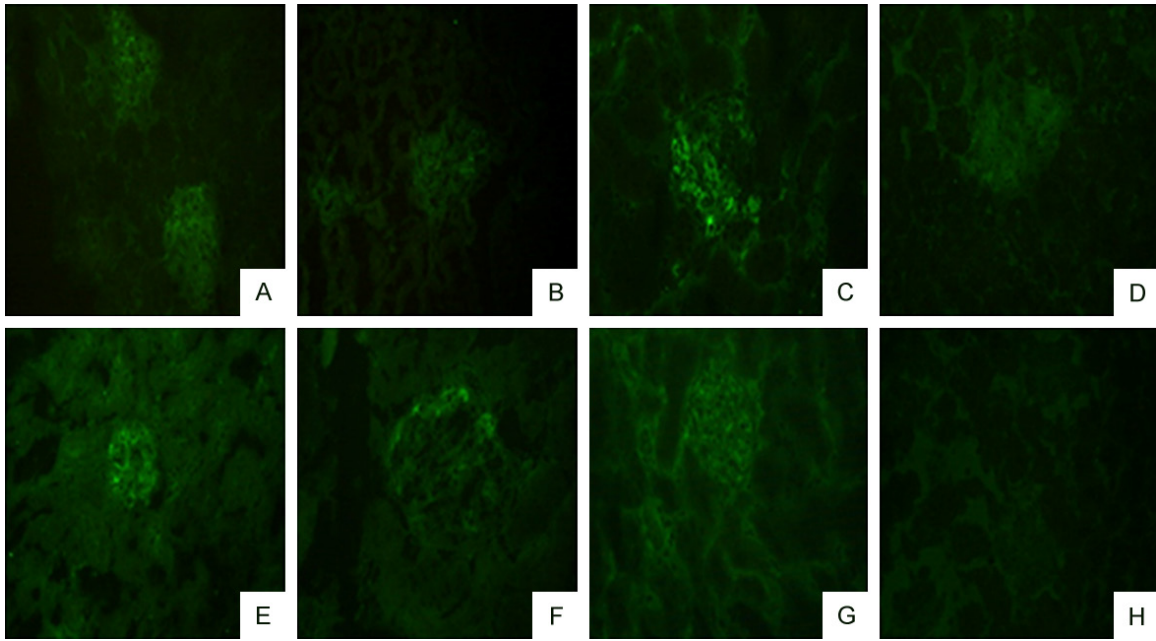


Figure 2. Detection of RSV-F protein in renal tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). (A) RSV₄; (B) RSV₈; (C) RSV₁₄; (D) RSV₃₀; (E) RSV₆₀; (F) RSV₉₀; (G) RSV₁₂₀; (H) Control group. Green fluorescence was detected in samples prepared from renal tissues (A-G), while no positive signal was detected in sample from normal control group (H).

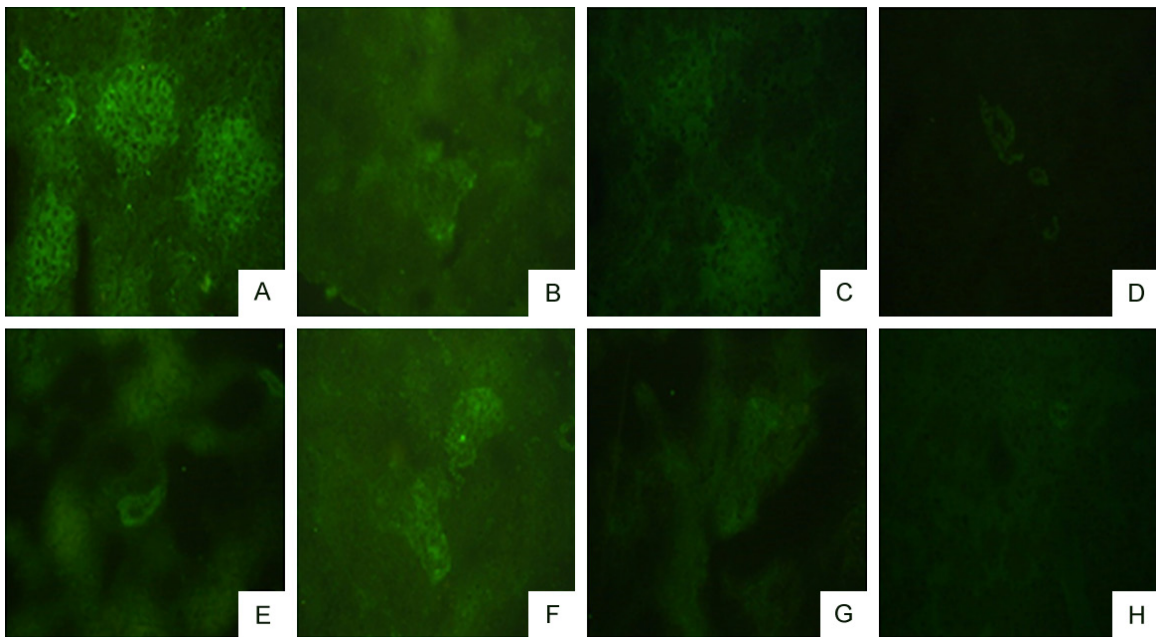


Figure 3. Detection of TLR3 protein (green fluorescence) in renal tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

marginal sinus and blood sinus. The intensity of immunofluorescence was different in each RSV infected group, especially both RSV₄ and RSV₈ groups showed obvious fluorescence, but RSV₃₀

and other groups presented gradually decreased fluorescence intensity. In control group, immunofluorescence staining results indicated there was no RSV-F protein expres-

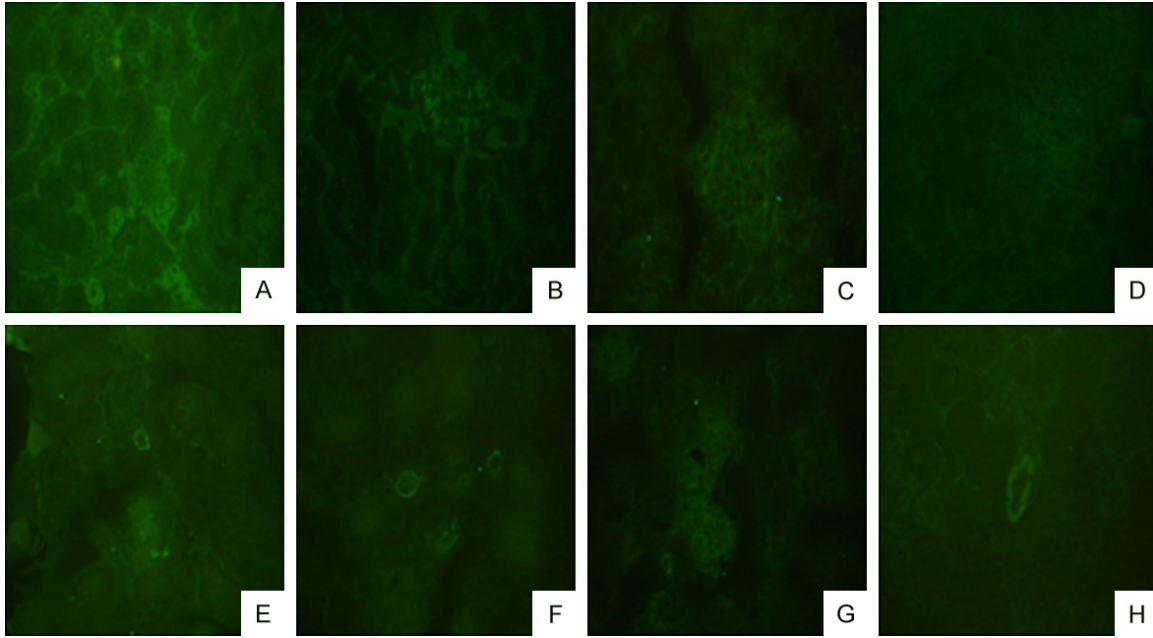


Figure 4. Detection of TLR4 protein in renal tissues (green fluorescence) of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

Table 5. The mRNA expressions of TLR3, TLR4, IFN- γ and IL-13 in kidney tissues of RSV inoculated rats of each group

Groups	$\Delta Ct (\bar{x} \pm s)$			
	TLR3 (n=5)	TLR4 (n=5)	IFN- γ (n=5)	IL-13 (n=5)
Control	7.167 \pm 0.577	16.167 \pm 0.764	13.333 \pm 0.764	21.333 \pm 1.155
RSV ₄	4.3 \pm 0.670*	14.5 \pm 1.354	10.5 \pm 0.912*	14.9 \pm 1.710*
RSV ₈	6.25 \pm 0.288	16.375 \pm 1.108	12 \pm 0.6123	20 \pm 2.274
RSV ₁₄	3.4 \pm 0.961*	16.5 \pm 2.121	10.5 \pm 0.707*	14 \pm 2.958*
RSV ₃₀	6.125 \pm 0.853	15.7 \pm 0.670	11.9 \pm 1.4317	20.5 \pm 1.903
RSV ₆₀	6.125 \pm 0.629	17.25 \pm 1.190	12.125 \pm 1.547	15 \pm 6.490*
RSV ₉₀	2.3 \pm 0.273*	14.9 \pm 1.431	13.375 \pm 2.286	12 \pm 0.790*
RSV ₁₂₀	4.833 \pm 0.752*	17.583 \pm 1.114	11.7 \pm 1.151	13.916 \pm 3.967*

*P<0.05, vs. Normal control.

Table 6. The content of TLR4 in PBMCs (% , $\bar{x} \pm s$)

Groups	N	Percentage (%)	P
Control	5	23.1 \pm 2.2	
RSV ₄	5	21.12 \pm 2.340	<0.05
RSV ₈	5	27.9 \pm 4.468	>0.05
RSV ₁₄	5	31.86 \pm 5.043	<0.05
RSV ₃₀	5	31.66 \pm 2.933	<0.05
RSV ₆₀	5	35.2 \pm 3.022	<0.05
RSV ₉₀	5	28.167 \pm 2.739	>0.05
RSV ₁₂₀	5	24.7 \pm 2.044	>0.05

tion in kidney, lung or spleen tissues of healthy rats.

Expressions of TLR3 and TLR4 in RSV infected rats

The expressions of TLR4 and TLR3 in kidney, lung and spleen tissues of RSV nephropathy rats was detected by Quantitative Real-time PCR and immunofluorescence staining respectively, and the expression of TLR4 in PBMCs was detected by flow cytometry. The results demonstrated that TLR3 and TLR4 were

expressed in both RSV infected and control groups (Figures 3, 4; Table 5). Briefly, compared with control group, TLR3 expression increased after RSV infection, particularly in RSV₄, RSV₁₄, RSV₉₀ groups, while TLR4 showed increase only in RSV₄ group. There was no significant difference between control group and other groups. To be specific, in kidney tissue, TLR3 and TLR4 were diffusely distributed in glomerulus and tubular with higher expression in glomerulus in RSV₄, RSV₁₄, RSV₉₀ groups (Figures 3, 4A-C), conversely, from Day 30, their expressions turned to focal distribution and more spread in

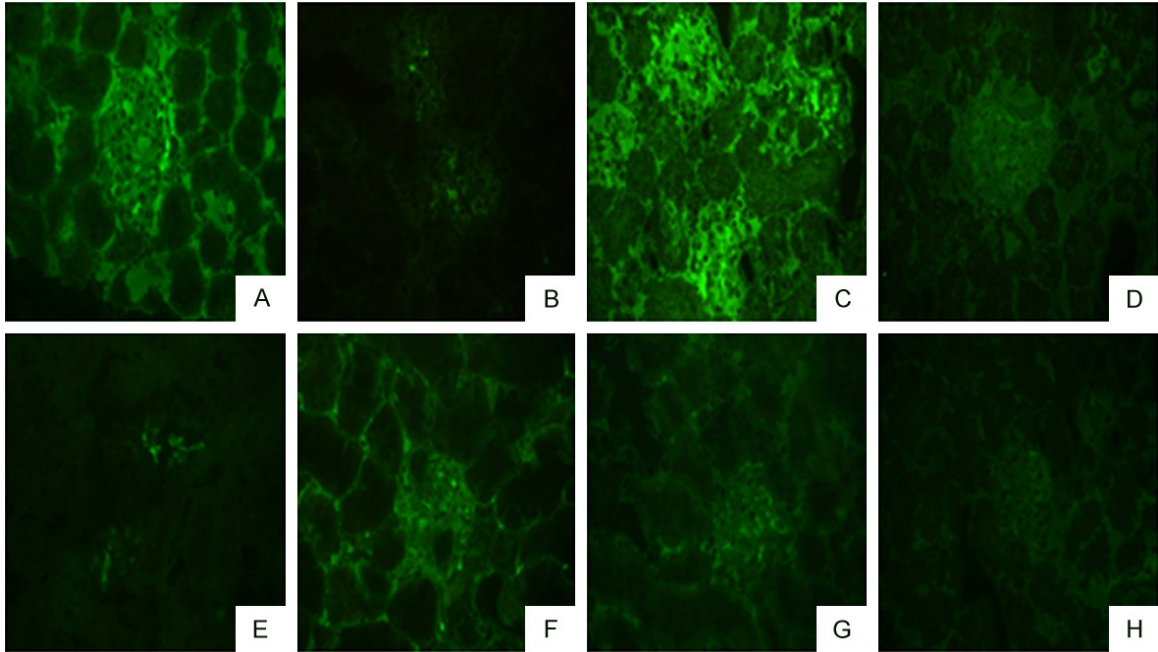


Figure 5. Detection of NF- κ B protein (green fluoresce) in renal tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

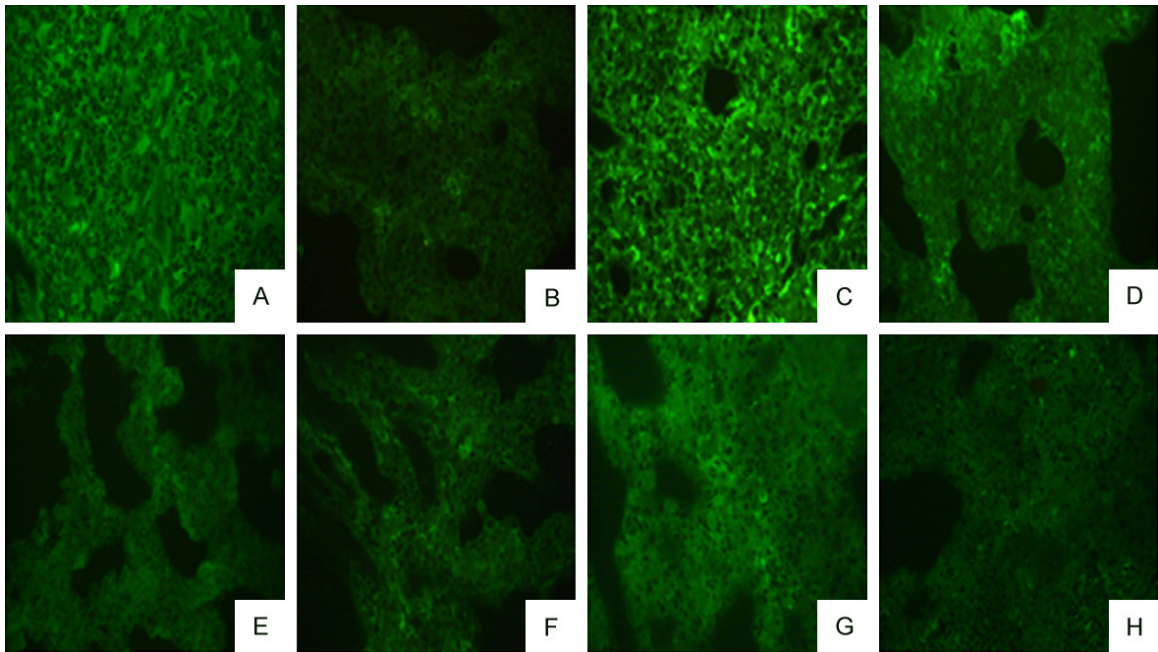


Figure 6. Detection of NF- κ B protein (green fluoresce) in lung tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

renal tubular (**Figures 3, 4D-G**); in lung tissues, TLR3 and TLR4 were also diffusely expressed in RSV₄, RSV₈, RSV₁₄ groups, and focally expressed on pulmonary bronchiole and epithelial cells in RSV₃₀ and other groups; in

spleen tissues, similarly, they were diffusely expressed and found mostly in marginal sinus and blood sinus. Especially, TLR3 expression was more evidently in kidney and lung tissues than in spleen tissues, while TLR4 was ex-

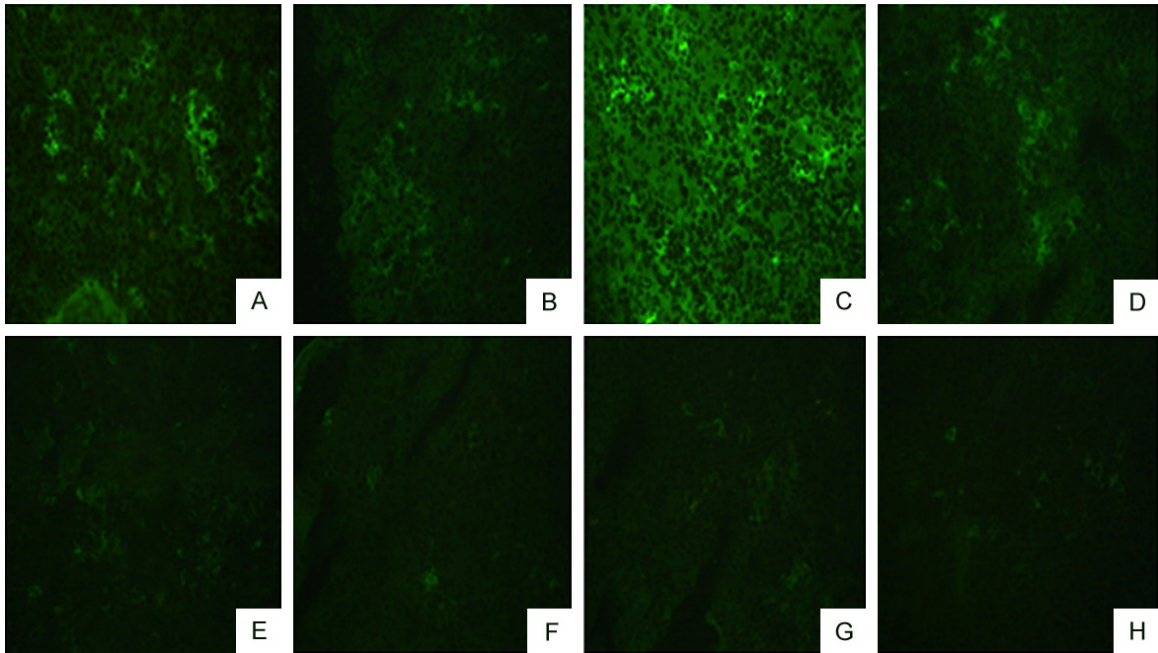


Figure 7. Detection of NF- κ B protein (green fluoresce) in spleen tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

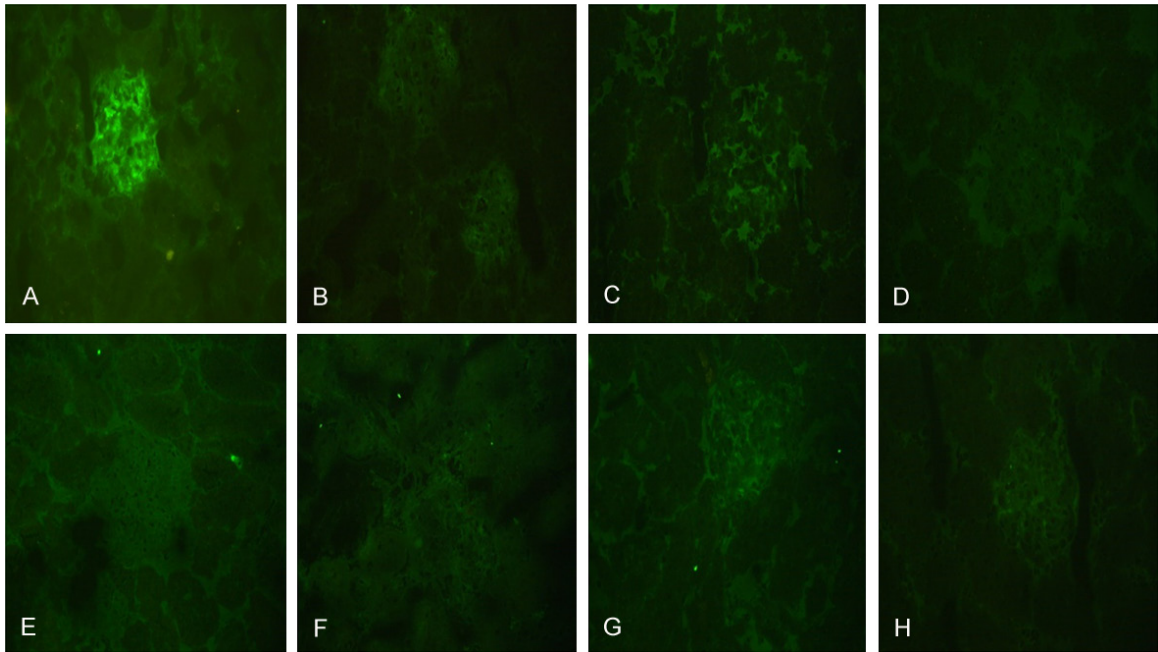


Figure 8. Detection of IFN- γ protein (green fluoresce) in renal tissues of RSV-inoculated rats by indirect immunofluorescence staining ($\times 400$). A: RSV₄; B: RSV₈; C: RSV₁₄; D: RSV₃₀; E: RSV₆₀; F: RSV₉₀; G: RSV₁₂₀; H: Control group.

pressed markedly in lung tissues than in kidney and spleen tissues. In addition, the expressions of TLR3 and TLR4 in PBMCs of each RSV infected group were significantly higher than those in control group (Table 6).

Expression of NF- κ B in rats with RSV-induced nephropathy

NF- κ B protein was observed in kidney, lung and spleen in all groups, however, its expression

Table 7. The expressions of IFN- γ in the kidney, lung, spleen and plasma of RSV inoculated rats of each group (pg/ml)

Groups	IFN- γ ($\bar{x} \pm s$)			
	Kidney (n=5)	Lung (n=5)	Spleen (n=5)	Plasma (n=5)
Control	878.62 \pm 59.68	205.52 \pm 29.71	116.07 \pm 12.48	12.60 \pm 1.61
RSV ₄	3132.61 \pm 88.47*	232.35 \pm 57.16	192.87 \pm 12.67*	22.89 \pm 2.15*
RSV ₈	1398.62 \pm 42.02	172.33 \pm 94.10	117.93 \pm 15.98	32.04 \pm 5.18*
RSV ₁₄	2742.06 \pm 57.89*	152.38 \pm 6.77	181.85 \pm 14.71*	18.42 \pm 3.29*
RSV ₃₀	958.07 \pm 23.21	101.45 \pm 23.59	103.70 \pm 32.07	13.44 \pm 2.39
RSV ₆₀	1099.12 \pm 32.32	144.19 \pm 37.93	141.45 \pm 42.98	12.84 \pm 4.73
RSV ₉₀	1289.20 \pm 45.23	168.10 \pm 46.48	106.89 \pm 24.56	7.66 \pm 0.41
RSV ₁₂₀	1009.26 \pm 22.68	219.28 \pm 29.48	149.53 \pm 21.59	11.91 \pm 5.79

*P<0.05, vs. control group.

Table 8. The expressions of IL-13 in the kidney, lung, spleen and plasma of RSV inoculated rats of each group (pg/ml)

Groups	IL-13 ($\bar{x} \pm s$)			
	Kidney (n=5)	Lung (n=5)	Spleen (n=5)	Plasma (n=5)
Control	240.49 \pm 11.11	29.33 \pm 2.61	2.89 \pm 1.83	7.11 \pm 1.80
RSV ₄	444.26 \pm 21.29*	25.09 \pm 1.87	8.90 \pm 1.14*	23.34 \pm 1.39*
RSV ₈	214.10 \pm 22.59*	29.65 \pm 2.42	3.12 \pm 1.07	6.82 \pm 2.21
RSV ₁₄	475.48 \pm 17.07*	41.55 \pm 5.39	8.32 \pm 2.12	22.70 \pm 2.72*
RSV ₃₀	233.95 \pm 31.74	23.80 \pm 5.01	4.13 \pm 1.67	7.04 \pm 3.81
RSV ₆₀	315.41 \pm 14.49	19.67 \pm 6.83	4.93 \pm 1.38	13.03 \pm 9.40*
RSV ₉₀	650.91 \pm 10.38*	21.36 \pm 5.04	8.59 \pm 3.17*	17.34 \pm 2.38*
RSV ₁₂₀	3225 \pm 26.13*	26.87 \pm 3.57	17.55 \pm 3.72*	11.96 \pm 2.49

*P<0.05, vs. control group.

level in RSV infected groups was higher than in control group (Figures 5-7), especially in RSV₄, RSV₁₄, RSV₉₀ and RSV₁₂₀ groups. The results indicated a diffuse distribution of NF- κ B protein in glomerulus and renal tubular as well as on lung tissues, while a focal spread in spleen tissues with more expression in marginal sinus and blood sinus.

Expression of IFN- γ and IL-13 in rats with RSV-induced nephropathy

The expression of IFN- γ and IL-13 in kidney, lung and spleen tissues as well as in plasma was detected by immunofluorescence staining and ELISA, respectively. The results showed that IFN- γ and IL-13 were all expressed in all the above tissues and plasma (Figure 8; Tables 7, 8). Generally speaking, compared with control group, IFN- γ level of RSV infected groups significantly increased on Day 8 and 14, then gradually decreased from Day 30, and there was no

significant difference between control group and RSV₉₀ and RSV₁₂₀ groups. Additionally, IFN- γ was diffusely expressed on glomerulus and renal tubular of kidney tissues (Figure 8A-G) and lung tissues, while focally expressed on spleen tissues with more distribution in marginal sinus and blood sinus. The IFN- γ expression in these three tissues showed no apparent difference.

As for IL-13, its expression was increased in all RSV infected groups. At the early stage, the IL-13 expression was gradually improved with an obvious increase on Day 4 and 14 (compared with control group, P<0.05), and then slowly decreased, while visibly increased again on Day 90. Moreover, its expression in lung tissues didn't change remarkably, however, it was noticeably enhanced on Day 4, 14 and 120 in spleen tissues and plasma (Table 8).

Correlation between the expressions of TLR3, TLR4, NF- κ B, IFN- γ , IL-13, 24 h proteinuria and RSV infection time as well as the pathological change of tissues

On day 4 and 8 after the inoculation, RSV was at high load level, the expression levels of TLR3, TLR4, NF- κ B, IFN- γ and IL-13 all showed observable increase, and the inflammation reaction in kidney tissues gradually improved and reached a peak on day 8, however, the proteinuria as well as podocyte construction had no remarkable change. From day 30, TLR3, NF- κ B and IL-13 increased as the RSV content increasing; but when RSV replicated at a low level, TLR3, NF- κ B and IL-13 levels decreased significantly while the expressions of TLR4 and IFN- γ had no obvious change compared to control group. Meanwhile, the inflammation reaction in kidney tissues was alleviated, however, the podocyte fusion and proteinuria level obviously increased (Figure 9).

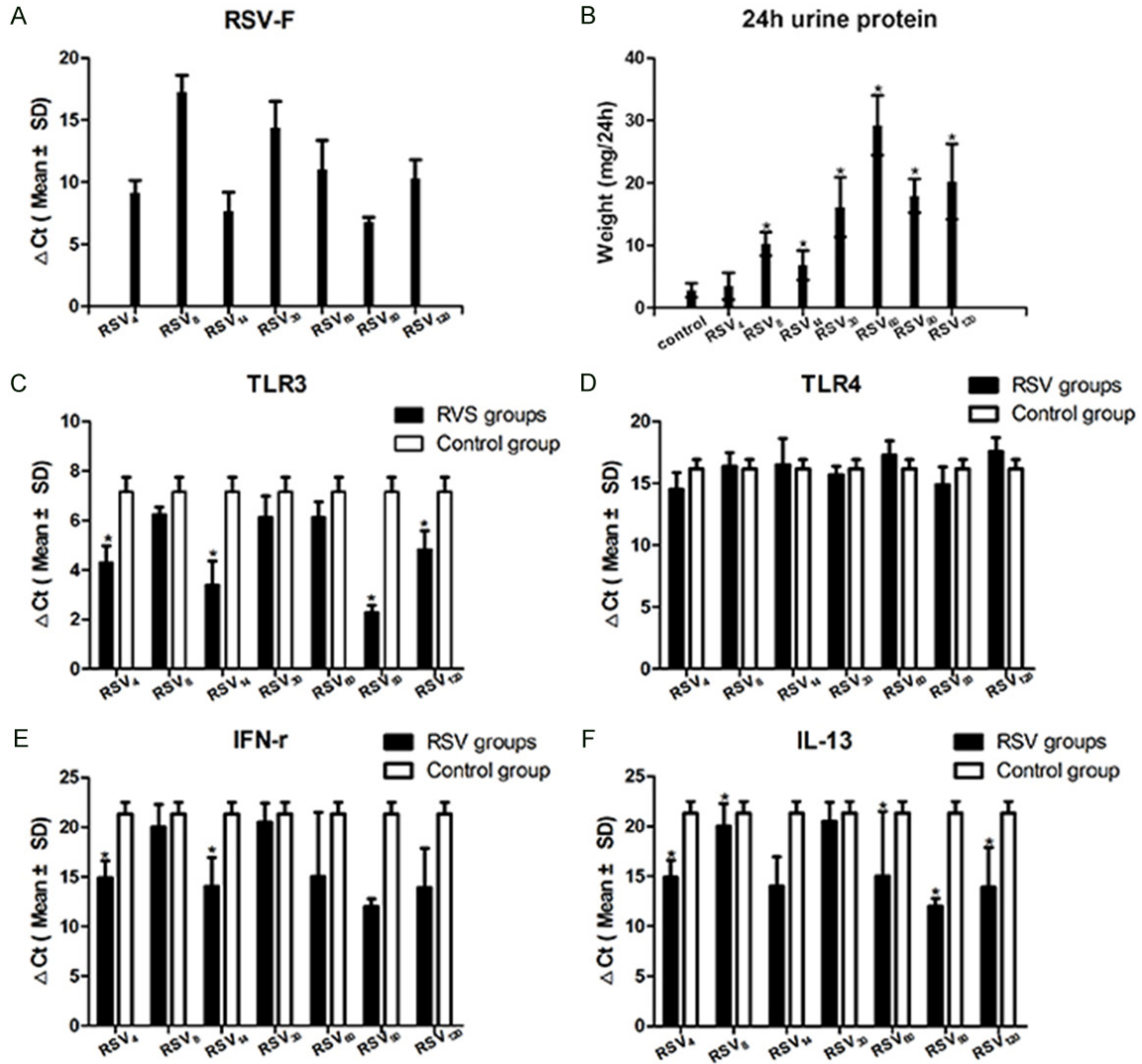


Figure 9. Correlation between the mRNA expressions of TLR3, TLR4, NF- κ b, IFN- γ , IL-13, 24 h proteinuria and RSV-F in in renal tissues of RSV-inoculated rats.

Discussion

TLR3 which mainly distributes on the immature dendritic cells and also expresses on T cell, NK cell, macrophages and mouse and human renal tubular epithelial cells and mesangial cells [11, 13], plays a key role in the antiviral response of host. However, by infecting human kidney 293 cell line via RVS and interfering TLR3 expression with siRNA, Rudd et al. [18] found that the virus load in infected was not obvious changed, indicating TLR3 had no effect on RSV replication.

Virus RNA can lead to the endogenous tolerance and exogenous antigens loss, thus result-

ing in the imbalance of Th1/Th2 in the specific immune response, which is related to the pathology of RSV infectious disease [19-22]. IFN- γ and IL-13 are the representatives of Th1, Th2 cytokines, respectively. On the one hand, IL-13 is closely related with the onset of MCNS, for example, Kin-Wai Lai et al. [23] used IL-13 to transfect rats, and found that high expression of IL-13 could cause MCNS in rats, presenting with podocytes impairment and fusion. In this study, we found that after RSV inoculation, the expressions of TLR3, NF- κ B and IL-13 were in the same trend with RSV expression. With the increase of virus load, TLR3 was stimulated to activate immune response and to promote the expression of IL-13. The correlation between

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TLR3, IL-33, 24 h proteinuria and podocyte fusion as well as other related pathological changes were summed and analyzed as follows.

At the acute infection phase in the early stage (day 4), high RSV load activated and up-regulated the expression of TLR3, which turned on the expression of IL-13, increased the expression of proteinuria, and led to unobvious fusion of podocytes. On day 8, most of the viruses were cleared, as a result, TLR3 expression as well as IL-13 significantly decreased nearly to the normal level, but the direct injury on kidney by virus still led to evidently increased level of proteinuria and renal morphological changes, such as swelling of glomerular endothelial cells and chromatin aggregation etc. On day 14, virus load increased again, so as to the expression of TLR3 as well as IL-13 was down-regulated after the recognition of RSV, nevertheless the proteinuria was alleviated, the podocyte fusion appeared. On Day 30, virus was cleared and TLR3 and IL-33 expressions were decreased, while the level of proteinuria increased significantly with the gradually severe podocyte fusion which peaked on Day 60. From Day 90, virus replication increased and produced dsRNA, which stimulated the expression of TLR3 and IL-13, similarly, proteinuria and podocyte fusion increased significantly as well. All of these findings were suspected to be due to the repeated increases of IL-13 and other cytokines induced by RSV activating NF- κ B, had already damaged the kidney, resulting in increased proteinuria and podocyte fusion. Moreover, TLR3 expression in kidney tissue was higher than that in lung and spleen, indicating TLR3 signaling pathway might be involved in the regulating of immune-injury of RSV on kidney. But on Day 8, 30, when RSV was at a low replication level, TLR3 and its signaling pathway were obviously inhibited, suggesting that RSV at low replication load may escape from TLR3 recognition and restrict antigen presentation and therefore the immune system couldn't be activated.

On the other hand, our research confirmed that the expression trend of IFN- γ was basically consistent with that of RSV-F protein in the tissues and plasma of infected rats, however, IFN- γ expression level only notably increased in the acute phase of infection, while it's expression showed no significant difference comparing to

the control group at the late phase, so IFN- γ level didn't change apparently with the increase of RSV-F, indicating that inhibition of IFN- γ expression might decrease the ability for body to clear the virus, thereby cause persistent RSV infection.

TLR4 which is widely expressed on various epithelial and endothelial cells including respiratory tract, genitourinary epithelial cells (podocytes, renal tubular epithelial cells) and etc, plays a vital role in the clearance of RSV infection, for example, a previous study has confirmed that RSV in TLR4-knockout mice could be replicated to a higher titer and sustained for a long time, and in this kind of rat, lung NK cells and CD14+ cells malfunction appeared, and the expression of IL-12 was insufficient after RSV infection, all of these could lead to poor virus clearance [24]. We found that the TLR4 expression increased in kidney, spleen and lung tissues at the early phase of RSV infection, then decreased, but at the late phase, the expression was increased again; however, the increase wasn't significant compared to the uninfected ones, indicating that TLR4 signaling pathway was inhibited. TLR4 mainly recognize RSV-F protein, which has two forms: immature F protein like split virion and mature F protein that expressed in virus particles and the surface of infected cells. Mature F protein can change to a new protein conformation to evade TLR4 recognition, thus escape from host immune surveillance which might be the mechanism of persistent presence of RSV in the kidney, spleen and lung tissues and the ineffective activation of TLR4 signaling pathway [21].

In conclusion, RSV may realize immune escape by interfering TLR signaling pathway and inducing Th2-based immune response to release multiple inflammatory cytokines such as IL-13 or some anti-inflammatory cytokines and chemokines which will lead the Th1/Th2 imbalance and further cause immune disorder of the host.

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Disclosure of conflict of interest

None.

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