

## Original Article

# Re-infection with respiratory syncytial virus aggravates renal injury

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**Abstract:** We previously reported that respiratory syncytial virus (RSV) and RSV-directed antibodies were detected in the respiratory tract epithelial cells, PBMC, serum, urine and renal tissue of patients with steroid-resistant nephrotic syndrome. In rats, high doses of RSV can increase urinary protein content, decrease serum albumin, and induce symptoms characteristic of human minimal change nephrotic syndrome (MCNS) in 14-28 days post-infection. Our study aimed to investigate the effect of re-infection with RSV on renal injury. Rats were inoculated with  $6 \times 10^4$  or  $6 \times 10^6$  PFU, then after 14 days re-inoculated with  $6 \times 10^4$  or  $6 \times 10^6$  PFU RSV, and sacrificed after a further 42 days. Serum levels of IL-6, IL-17, and TGF- $\beta$ , proteinuria, urinary glycosaminoglycans (GAGs) were measured and histopathologic changes and RSV F protein content of the kidney was assessed by electron microscopy and *in situ* hybridization. Proteinuria was higher following RSV re-infection than primary infection, and was accompanied by hypoproteinemia. Podocyte damage was more extensive after re-infection than primary infection, and in mice reinfected with  $6 \times 10^6$  PFU RSV mesangial cell and mesangial matrix proliferation was observed. Serum levels of IL-6 and IL-17 were significantly higher following re-infection with RSV, and were positively associated with proteinuria. Excretion of urinary GAGs was higher following re-infection with RSV, and RSV F protein was expressed in the late stage of RSV infection. RSV re-infection induces higher proteinuria and more serious renal damage than primary RSV infection. RSV may directly damage the kidney, and during relapse immune dysfunction may exacerbate the renal pathology.

**Keywords:** Respiratory syncytial virus, nephrosis, F protein, human respiratory syncytial virus, immunopathogenesis, proteinuria

## Introduction

Minimal change nephrotic syndrome (MCNS), a disease of the kidney most commonly seen in young children, can cause nephrotic syndrome, nonspecific damage to the kidney characterized by proteinuria, which can cause hypoalbuminemia. Although MCNS is sensitive to glucocorticoids, is often recurs, and viral infections can contribute to MCNS. In 1986 MacDonald *et al.* reported that Respiratory Syncytial Virus (RSV) could aggravate primary nephrotic syndrome [1].

RSV was the most commonly identified respiratory track viruses in children with steroid-sensitive and simple nephrotic syndrome [2-4]. Kawasaki *et al.* conducted a follow-up study of patients with RSV infection and reported that the severity of illness attributed to RSV re-infection was generally milder than that in primary infection [5]. Although a study of RSV infection

in children in Kenya found immunity to RSV re-infection was partial and short lived [6], animal models of RSV re-infection have implicated T cell responses in exacerbated disease [7]. Tregoning *et al.* had reported that T cells play a central role in the outcome of neonatal RSV infection, and can act to exacerbate disease during RSV re-infection [8]. We previously found that RSV and RSV-directed antibodies were present in the respiratory tract epithelial cells, PBMC, serum, urine and renal tissue of patients with steroid-resistant nephrotic syndrome, and that exacerbation and relapse of the primary nephrotic syndrome was closely related to respiratory infection, making treatment of both conditions difficult. Additionally, we also found that the levels of RSV-directed antibodies in the serum were positively associated with proteinuria during steroid responsive nephrotic syndrome [4, 9, 10]. However whether RSV-induced proteinuria is more severe during re-infection remains to be determined.

## RSV re-infection may directly and/or indirectly aggravate renal damage

We sought to investigate the influence of the RSV re-infection on proteinuria and glomerular structure in rats. Of the 11 proteins the negative single-strand RNA virus RSV encodes, the fusion protein (F) is a transmembrane surface glycoproteins mediating RSV attachment to cells [11]. We measured expression of RSV F protein mRNA in the kidney of RSV-inoculated rats, and explored the effect of RSV re-infection on symptoms of nephrotic syndrome.

### Materials and methods

#### *Viruses and cells*

The long strain of Human RSV and the HeLa cell line were obtained from the Viral Institute of the Chinese Academy of Preventive Medical Science. RSV was cultured in HeLa cells and assayed for infectivity [12]. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Invitrogen, USA) containing 10% fetal calf serum at 37°C in 5% CO<sub>2</sub>. RSV was propagated in HeLa cells in a monolayer culture. RSV titer was determined by a methylcellulose plaque assay.

#### *Animals*

Specific pathogen free male Sprague-Dawley rats, weighing 180-200 g (Da Shuo company, Sichuan, Chengdu, China) were divided into 5 groups of 5 rats each. One group (group A) was inoculated with 6×10<sup>6</sup> PFU RSV intranasally (0.2 ml) and intraperitoneally (0.4 ml) daily for three days. Three groups (groups B, C, and D) were inoculated with 6×10<sup>4</sup> PFU RSV intranasally (0.2 ml) and intraperitoneally (0.4 ml) daily for three days. Group E rats were inoculated with virus-free Dulbecco's modified Eagle's medium (DMEM). After 14 days, groups A, B and E rats were re-inoculated with DMEM, and groups C and D were re-inoculated with 6×10<sup>6</sup> PFU, or 6×10<sup>4</sup> PFU RSV, respectively, intranasally (0.2 ml) and intraperitoneally (0.4 ml) daily for three days. All rats were sacrificed on the 56<sup>th</sup> day after primary infection.

Measurement of proteinuria, urinary glycosaminoglycans (GAGs) excretion and serum parameters.

On the day before rats were sacrificed, urine was collected over 24 h and protein content was measured by pyrogallol end-point method. On the day before rats were sacrificed, Urinary GAGs excretion was examined by the improved

Whiteman process [13, 14]. Urinary GAGs was measured by reference to a calibration curve constructed using a standard preparation of heparin sulfate, and corrected for urinary creatinine.

The blood was collected from the hearts of rats on the 56<sup>th</sup> day after primary infection and serum albumin, cholesterol, urea nitrogen and creatinine were measured (Hitachi 7600; Hitachi, Tokyo, Japan). The levels of IL-6, IL-17, TGF-β in serum were detected by enzyme linked immunosorbent assay (R&D System, Minneapolis, US).

#### *Histopathologic studies*

The shape and the weight of the whole kidney were observed. Renal tissue (0.5 cm×0.7 cm) was fixed in 4% paraformaldehyde at 4°C for 24 h and embedded in paraffin. Paraffin sections (4 μm) were used for haematoxylin-eosin staining. The expression of RSV F protein mRNA was detected by *in situ* hybridization and integrated optical density (IOD) was analyzed using ImageJ software (NIH, Bethesda, MD). Fresh renal tissue was fixed in 3% glutaral phosphate buffer for ultrastructure analysis by electron microscope (H-600IV transmission electron microscope, Hitachi).

#### *Statistical analysis*

Data are expressed as means ± SD. For multiple comparison, the Bonferroni or DunnettT3 test that were used. P<0.05 was considered significant. Statistical analysis was carried out with the Statistical Package for the Social Sciences ver.16.0.

### Results

#### *Proteinuria*

To investigate the effects of RSV infection on the kidney, rats were inoculated with 6×10<sup>6</sup> or 6×10<sup>4</sup> PFU RSV or vehicle control, and after 14 days some rats inoculated with 6×10<sup>4</sup> PFU RSV were re-inoculated with 6×10<sup>4</sup> or 6×10<sup>6</sup> PFU RSV, and all other animals were most re-inoculated with vehicle. As indicated in **Table 1**, after secondary inoculation proteinuria was measured, and found to be higher in rats initially inoculated with 6×10<sup>6</sup> or 6×10<sup>4</sup> PFU RSV than in rats inoculated with vehicle (5.627±1.038 mg/24 h; P<0.05), and was significantly higher in rats inoculated with 6×10<sup>6</sup> PFU RSV than

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**Table 1.** 24 hour Proteinuria and serum parameters (mean  $\pm$  standard deviation)

Groups (n=5)	Proteinuria (mg/24 h)	Albumin (g/L)	BUN (mmol/L)	Scr ( $\mu$ mol/L)	TC (mmol/L)
A	27.047 $\pm$ 2.479 <sup>*,<math>\Delta</math></sup>	16.42 $\pm$ 1.532 <sup>*,#<math>\Delta</math></sup>	31.6 $\pm$ 7.021	8.60 $\pm$ 1.787	1.62 $\pm$ 0.314
B	10.139 $\pm$ 1.404 <sup>*,#</sup>	21.44 $\pm$ 2.492 <sup>#</sup>	25.4 $\pm$ 4.722	6.94 $\pm$ 1.181	1.404 $\pm$ 0.353
C	36.052 $\pm$ 4.608 <sup>*,#<math>\Delta</math></sup>	15.72 $\pm$ 1.619 <sup>*,<math>\Delta</math></sup>	33.0 $\pm$ 8.515	8.04 $\pm$ 3.503	1.63 $\pm$ 0.467
D	13.817 $\pm$ 1.635 <sup>*,#<math>\Delta</math></sup>	19.64 $\pm$ 2.964 <sup>*,#</sup>	31.4 $\pm$ 7.127	9.97 $\pm$ 4.235	1.48 $\pm$ 0.285
E	5.627 $\pm$ 1.038	22.98 $\pm$ 3.080	29.4 $\pm$ 6.949	6.30 $\pm$ 1.801	1.28 $\pm$ 0.1178

A,  $6 \times 10^6$  PFU RSV primary infection; B,  $6 \times 10^4$  PFU RSV primary infection; C,  $6 \times 10^4 + 6 \times 10^6$  PFU RSV; D,  $6 \times 10^4 + 6 \times 10^4$  PFU RSV; E, control group. <sup>\*</sup>P<0.05, vs. Group E; <sup>#</sup>P<0.05, vs. Group A;  <sup>$\Delta$</sup> P<0.05, vs. Group B; BUN, Blood Urea Nitrogen; Scr, serum creatinine; TC, total cholesterol.

**Table 2.** Levels of serum IL-6, IL-17, TGF- $\beta$ , GAGs and F protein (mean  $\pm$  SD)

Group (n=5)	IL-6 (pg/ml)	IL-17 (pg/ml)	TGF- $\beta$ (pg/ml)	F protein (IDO)	GAGs (mg/mmolCr)
A	129.11 $\pm$ 6.500 <sup>*,#</sup>	1029.40 $\pm$ 87.818 <sup>*</sup>	771.71 $\pm$ 51.411 <sup>*,<math>\Delta</math></sup>	3442.87 $\pm$ 525.685	36.33 $\pm$ 5.556 <sup>*,#<math>\Delta</math></sup>
B	84.89 $\pm$ 7.316 <sup>*,#</sup>	554.20 $\pm$ 54.995 <sup>*,#</sup>	376.85 $\pm$ 35.164 <sup>*,#</sup>	3925.76 $\pm$ 726.656	24.77 $\pm$ 5.359 <sup>*,#</sup>
C	290.11 $\pm$ 16.458 <sup>*,#<math>\Delta</math></sup>	1752.60 $\pm$ 132.813 <sup>*,#<math>\Delta</math></sup>	881.62 $\pm$ 24.338 <sup>*,#<math>\Delta</math></sup>	3762.36 $\pm$ 928.613	57.74 $\pm$ 8.567 <sup>*,#<math>\Delta</math></sup>
D	171.85 $\pm$ 9.335 <sup>*,#<math>\Delta</math></sup>	757.36 $\pm$ 44.348 <sup>*,#<math>\Delta</math></sup>	390.13 $\pm$ 15.377 <sup>*,#</sup>	3316.24 $\pm$ 622.097	32.98 $\pm$ 7.171 <sup>*,<math>\Delta</math></sup>
E	32.836 $\pm$ 6.748	175.52 $\pm$ 17.906	214.76 $\pm$ 6.905	No	8.482 $\pm$ 1.281

A,  $6 \times 10^6$  PFU RSV primary infection; B,  $6 \times 10^4$  PFU RSV primary infection; C,  $6 \times 10^4 + 6 \times 10^6$  PFU RSV; D,  $6 \times 10^4 + 6 \times 10^4$  PFU RSV; E, control group <sup>\*</sup>P<0.05, vs. Group E; <sup>#</sup>P<0.05, vs. Group A;  <sup>$\Delta$</sup> P<0.05, vs. Group B.

$6 \times 10^4$  PFU RSV (27.047 $\pm$ 2.479 mg/24 h and 10.139 $\pm$ 1.404 mg/24 h, respectively; P<0.05). Proteinuria increased following re-inoculation with  $6 \times 10^6$  or  $6 \times 10^4$  PFU RSV (P<0.05), and, again, was significantly higher in rats re-inoculated with  $6 \times 10^6$  than  $6 \times 10^4$  PFU RSV (36.052 $\pm$ 4.608 mg/24 h and 13.817 $\pm$ 1.635 mg/24 h, respectively; P<0.05).

### Urinary GAG excretion and Serum parameters

As indicated in **Table 1**, after secondary inoculation serum albumin levels were found to be significantly lower in rats initially inoculated with  $6 \times 10^6$  PFU RSV than in rats inoculated with vehicle (22.98 $\pm$ 3.080 g/L; P<0.05), and was significantly lower in rats inoculated with  $6 \times 10^6$  PFU RSV than  $6 \times 10^4$  PFU RSV (16.42 $\pm$ 1.532 g/L and 21.44 $\pm$ 2.492 g/L, respectively; P<0.05). Serum albumin levels decreased further following re-inoculation with  $6 \times 10^6$  or  $6 \times 10^4$  PFU RSV (15.72 $\pm$ 1.619 g/L and 19.64 $\pm$ 2.964 g/L, respectively; P<0.05).

The level of urinary GAG excretion was higher in rats initially inoculated with  $6 \times 10^6$  or  $6 \times 10^4$  PFU RSV than in rats inoculated with vehicle (8.482 $\pm$ 1.281 mg/mmolCr; P<0.05), and was significantly higher in rats inoculated with  $6 \times 10^6$  PFU RSV than  $6 \times 10^4$  PFU RSV (36.33 $\pm$ 5.556 mg/mmolCr and 24.77 $\pm$ 5.359 mg/mmolCr, respectively; P<0.05). GAG excretion increased

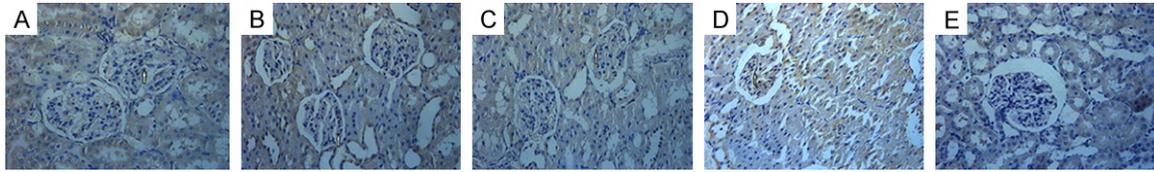
following re-inoculation with  $6 \times 10^6$  or  $6 \times 10^4$  PFU RSV (P<0.05), and, again, was significantly higher in rats re-inoculated with  $6 \times 10^6$  than  $6 \times 10^4$  PFU RSV (57.74 $\pm$ 8.567 mg/mmolCr and 32.98 $\pm$ 7.171 mg/mmolCr, respectively; P<0.05) (**Table 2**).

### RSV F protein expression in rat kidneys

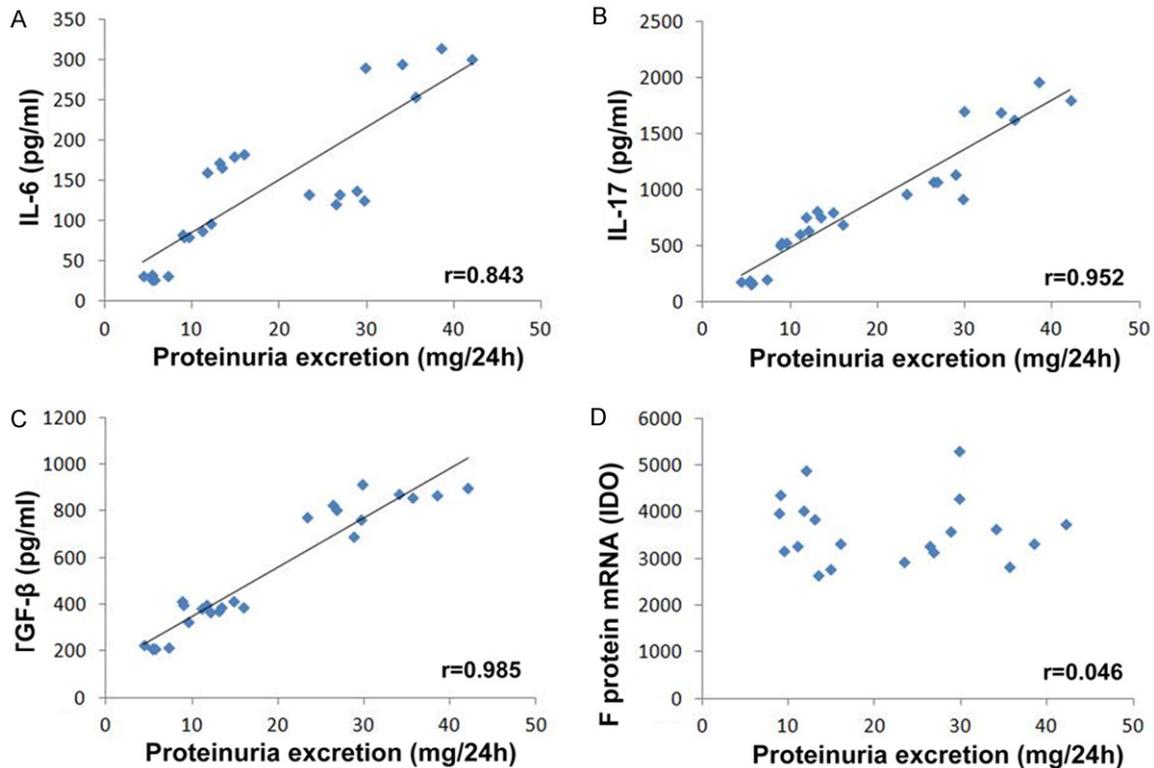
The expression of RSV F protein mRNA was detected using in situ hybridization. Whilst no F protein was detected in control animals, F protein was detected in the epithelial cells, mesangial cells of glomerulus and tubular epithelial cells of all infected animals. The IDO of RSV F protein mRNA signals did not differ significantly between animals infected with RSV as only a primary infection groups and those re-infected (IDO P>0.05; **Figure 1**).

The levels of serum IL-6, IL-17 and TGF- $\beta$  were higher in rats initially inoculated with  $6 \times 10^6$  PFU RSV (129.11 $\pm$ 6.500 pg/ml, 1029.40 $\pm$ 87.818 pg/ml and 771.71 $\pm$ 51.411 pg/ml, respectively) than rats inoculated with  $6 \times 10^4$  PFU RSV (84.89 $\pm$ 7.316 pg/ml, 554.20 $\pm$ 54.995 pg/ml and 376.85 $\pm$ 35.164 pg/ml, respectively), and higher still than in rats inoculated with vehicle (28.11 $\pm$ 8.047 pg/ml, 175.52 $\pm$ 17.906 pg/ml and 214.76 $\pm$ 6.905 pg/ml, respectively; all P<0.05). The levels of serum IL-6, IL-17 and TGF- $\beta$  were again significantly higher in rats re-

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**Figure 1.** Glomeruli from RSV primary infection and re-infection SD rats (In situ hybridization,  $\times 400$ ). The expression of RSV F protein mRNA was detected using in situ hybridization. Positive hybridization signals appeared in the epithelial cells, mesangial cells of glomerulus and tubular epithelial cells as indicated with white arrows.



**Figure 2.** Association between level of serum cytokines and F protein mRNA (IDO) with proteinuria. A: The relationship between the level of IL-6 and proteinuria ( $r=0.843$ ,  $P<0.05$ ); B: The relationship between the level of IL-17 and proteinuria ( $r=0.952$ ,  $P<0.05$ ); C: The relationship between the level of TGF- $\beta$  and proteinuria ( $r=0.958$ ,  $P<0.05$ ); D: The relationship between the level of F protein mRNA (IDO) and proteinuria ( $r=0.046$ ,  $P>0.05$ ).

inoculated with  $6 \times 10^6$  PFU RSV ( $290.11 \pm 16.458$  pg/ml,  $1752.60 \pm 132.813$  pg/ml and  $881.62 \pm 24.338$  pg/ml) and  $6 \times 10^4$  PFU RSV ( $171.85 \pm 9.335$  pg/ml,  $757.36 \pm 44.348$  pg/ml and  $390.13 \pm 15.377$  pg/ml; all  $P<0.05$ ) (Table 2 and Figure 2).

### Histopathology of kidneys

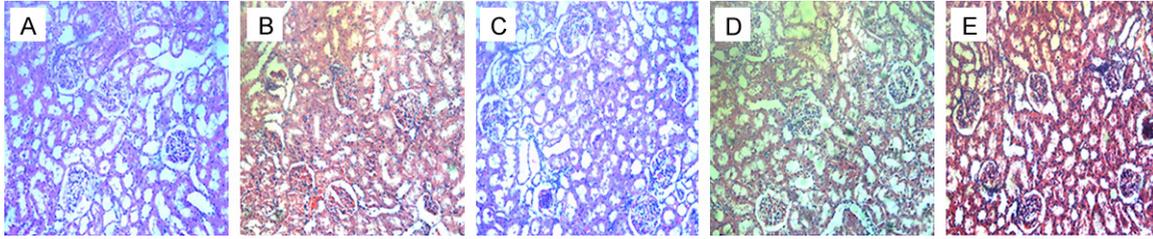
As illustrated in Figure 3, the kidneys of rats inoculated with  $6 \times 10^4$  or  $6 \times 10^6$  PFU RSV were swollen, cystic damage was only observed in the kidneys of rats re-inoculated with  $6 \times 10^4$  or  $6 \times 10^6$  PFU RSV. Using light microscopy the glomerulus of all rats appeared normal. Hy-

peremia and slight inflammatory cell infiltration was observed in the renal interstitium of all RSV inoculated rats. The tubular epithelial cells exhibited vacuolar degeneration and were swollen in rats inoculated or re-inoculated with  $6 \times 10^6$  PFU RSV or re-inoculated with  $6 \times 10^4$  PFU RSV. The extent and range of renal tubular changes was more notable in RSV re-infection than that in the RSV primary infection groups.

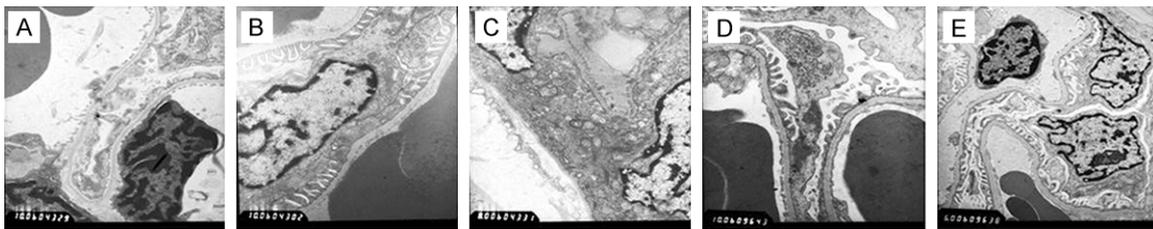
### Electron microscopy

Electron microscopy revealed podocyte damage in rats inoculated with  $6 \times 10^6$  PFU RSV. 75% of podocytes were damaged, and some renal

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**Figure 3.** Glomeruli from RSV primary infection and re-infection SD rats (HE,  $\times 200$ ). Representative images of Glomeruli from RSV inoculated rats, viewed by light microscopy. A: group A. B: group B. C: group C. D: group D. E: group E. In groups A and C Renal tubules were diffuse and vacuolar and granular degeneration was observed, while the renal interstitium was severely swollen. In group D, little diffuse vacuolar and granular degeneration was observed. In group B the tubular epithelia cells exhibited slight granular degeneration. No changes in the glomerulus of any groups were observed.



**Figure 4.** Renal tissue ultrastructure. A: Podocytes were preponderantly damaged (group A,  $\times 6000$ ); B: Podocytes were slightly damaged (group B,  $\times 6000$ ); C: Significant podocyte damage was accompanied by mesangial cell and mesangial matrix proliferation (group C,  $\times 8000$ ); D: Extensive podocyte damage (group D,  $\times 5000$ ). E: Normal control groups (group E,  $\times 8000$ ).

tubular epithelia cells were swollen and contained lipid deposits (**Figure 4A**). Little swelling and separation of podocytes was observed in rats inoculated with  $6 \times 10^4$  PFU RSV (**Figure 4B**). In rats re-inoculated with  $6 \times 10^6$  PFU RSV, podocytes were almost completely fused, and mesangial cell and mesangial matrix proliferation was observed (**Figure 4C**). In rats re-inoculated with  $6 \times 10^4$  PFU RSV podocyte fusion was aggravated (**Figure 4D**). No podocyte fusion was observed in control rats (**Figure 4E**), and no electron-dense deposits were observed in any of the groups (**Figure 4**).

### Discussion

We previously observed that rats infected with RSV exhibited proteinuria, which was accompanied by extensive podocyte damage, and minor changes in the mesangial cells and renal tubular epithelia cells. It was not known, however, whether RSV re-infection aggravated proteinuria and renal injury. As the titer of RSV in the pharynx nasalis of infected children was found to range between  $10^{3.5} \text{TICD}_{50}$  and  $10^6 \text{TICD}_{50}$  during respiratory infection, we infected rats with  $6 \times 10^4$  PFU or  $6 \times 10^6$  PFU RSV suspension.

Here we observed that rats infected with  $6 \times 10^4$  PFU RSV exhibited proteinuria, but serum albumin levels were not elevated in these animals, glomeruli were segmentally damaged and podocytes were damaged. Moreover, primary infection with high titers of RSV ( $6 \times 10^6$  PFU) induced pathological changes within the kidney and reduced serum albumin levels, mimicking the pathological changes observed MCNS children. Our results support the previous findings of Liu *et al.* [14]. Liu *et al.* observed that  $6 \times 10^6$  PFU RSV could increase urinary protein and decrease serum albumin in rats, but no changes were observed in podocytes of animals inoculated with  $6 \times 10^4$  PFU RSV. However, there was no information regarding the relationship between RSV re-infection and the proteinuria of rats.

Furthermore, symptoms of RSV infection had disappeared in the later stage of infection (the 56<sup>th</sup> day), and proteinuria increased. Elevated proteinuria and depressed serum albumin levels were more pronounced after re-infection than after initial infection. Re-infection with higher RSV titers induced more pronounced protein excretion than re-infection with lower

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RSV titers. RSV re-infection appeared to aggravate renal damage, suggesting that repeated infection may exacerbate nephrotic syndromes and make the treatment of this aspect of the disease more difficult. However, whether the mechanisms were responsible for this damage was unknown.

We hypothesized that RSV re-infection aggravated renal damage by directly destroying the glomerular filtration barrier. We found that excretion of urinary GAGs was increased more substantially after re-infection than primary infection and excretion of urinary GAGs was higher in rats inoculated with higher doses. Hallak *et al.* and Schmidtke *et al.* reported that cell surface GAGs, and heparin sulfates were necessary for RSV infection [15, 16]. The glomerular filtration barrier contains negative charge-GAGs chains, and, what's more, Guo *et al.* reported that low-molecular-weight heparin could alleviate proteinuria in rats through inhibiting RSV from binding with HS [17]. These observations suggest that RSV may cause direct renal injury by damaging the glomerular filtration barrier. Expression of RSV mRNA in the kidney further supports the theory that RSV causes direct kidney damage. However, we found that proteinuria in RSV infected rats did not entirely coincide with detection of RSV F protein mRNA or urinary GAGs excretion, suggestion that additional mechanisms may contribute to RSV-induced nephrotic damage.

We further investigated the relationship between rat immune responses to RSV and nephrotic damage. Serum levels of the cytokines IL-6, IL-17 and TGF- $\beta$  were elevated following RSV infection, and serum levels of IL-6 and IL-17 were higher following RSV re-infection than primary infection. These results suggested that nephrotic damage may be worsened by pathological adaptive immune responses to RSV. RSV infection induces cytokines including type I IFN and IFN- $\gamma$  [18-20], IL-6, IL-8, IL-10, IL-13 and IL-17 [21-24]; and chemokines including CCL3, CCL2, and CCL5 [25, 26]. Turner *et al.* reported that cytokines, including IL-17, IL-6, and IL-21, could bind with receptors on the mesangial cells and renal tubular epithelial cells, inducing chemokine release and recruitment of neutrophils and monocytes to the kidney. These processes could triggers pathological injury of kidney [27]. Hou *et al.* reported that Th17 cells also play a key role in persistent viral infection [28], and we observed that serum lev-

els of IL-6 and IL-17 were higher following RSV re-infection than primary infection. These results suggest that immunolesions maybe play a role in the nephrotic damage caused by RSV re-infection. Multiple studies have implicated the interaction of transforming growth factor  $\beta$  (TGF- $\beta$ ) with phosphatidylinositol-3-kinase (PI3K) in promotion of mesangial cell dysfunction and renal epithelial-to-mesenchymal transition [29-31]. What's more, Several studies also had implicated TGF- $\beta$  in the tight balance between survival and apoptotic responses in podocytes [32]. In this study we also found that serum TGF- $\beta$  levels were elevated after RSV infection, and were highest following re-infection with  $6 \times 10^6$  PFU. These results suggest that both immune injury and the direct action of RSV proteins may contribute to the pathogenesis of nephrotic syndrome.

Our study is the first to report an association between repeated RSV infection and relapse or aggravation of nephropathy-like syndrome in rats. Repeated RSV infection can affect renal pathology through both direct damage and immune injury, highlighting a potential new focus for treatment and prevention of relapse of nephrotic syndrome.

### Disclosure of conflict of interest

None.

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## RSV re-infection may directly and/or indirectly aggravate renal damage

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