

# Induction monotherapy with sirolimus has selected beneficial effects on glomerular and tubulointersititial injury in nephrotoxic serum nephritis

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**Background:** The study aimed to test the hypothesis that therapeutic treatment with a mammalian target of rapamycin complex 1 inhibitor reduces renal cell proliferation and attenuates glomerular and tubulointerstitial injury in the early phase of nephrotoxic serum nephritis (NSN) in rats.

**Methods:** Male Wistar-Kyoto rats received a single tail-vein injection of sheep anti-rat glomerular basement membrane serum (day 0) and were treated with vehicle or sirolimus (0.25 mg/kg/day by subcutaneous injection) from day 1 until day 14.

**Results:** Treatment with sirolimus attenuated kidney enlargement by 41% ( $P<0.05$ ), improved endogenous creatinine clearance by 50% ( $P<0.05$ ), and reduced glomerular and tubulointerstitial cell proliferation by 53% and 70%, respectively, ( $P<0.05$  compared to vehicle) in rats with NSN. In glomeruli, sirolimus reduced segmental fibrinoid necrosis by 69%, autologous rat immunoglobulin G deposition, glomerular capillary tuft enlargement, and periglomerular myofibroblast ( $\alpha$ -smooth muscle actin-positive cells) accumulation (all  $P<0.05$ ) but did not significantly affect glomerular crescent formation ( $P=0.15$ ), macrophage accumulation ( $P=0.25$ ), or the progression of proteinuria. In contrast, sirolimus preserved tubulointerstitial structure and attenuated all markers of injury (interstitial ED-1- and  $\alpha$ -smooth muscle actin-positive cells and tubular vimentin expression; all  $P<0.05$ ). By immunohistochemistry and Western blot analysis, sirolimus reduced the glomerular and tubulointerstitial expression of phosphorylated (Ser 235/236) S6-ribosomal protein ( $P<0.05$ ).

**Conclusion:** Induction monotherapy with sirolimus suppressed target of rapamycin complex 1 activation, renal cell proliferation, and injury during the early stages of rodent NSN, but the degree of histological protection was more consistent in the tubulointerstitium than the glomerular compartment.

**Keywords:** glomerulonephritis, proliferation, crescentic, rapamycin, inflammation, kidney

## Introduction

Crescentic glomerulonephritis (CGN) is a pathological pattern of glomerular injury in which a severe nephritogenic assault is initiated against the glomerular capillary wall due to autoantibody formation, immune complex deposition, or cell-mediated injury.<sup>1,2</sup> The acute renal pathological hallmarks of CGN are glomerular fibrinoid necrosis and crescent formation which, without treatment, evolves into glomerulosclerosis, tubulointerstitial fibrosis, and end-stage kidney failure.<sup>3</sup> The conventional therapy of CGN involves treatment with a combination cyclophosphamide and glucocorticoids, with or without plasmapheresis.<sup>4,5</sup> Although these therapies are effective in inducing

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remission, when commenced before the onset of advanced renal impairment, they are associated with significant systemic toxicity and do not specifically target the renal cellular pathogenesis of CGN.<sup>6,7</sup>

Immune-mediated glomerular capillary wall injury and formation of fibrin in Bowman's capsule triggers excessive glomerular cell proliferation and inflammation, resulting in the formation of cellular glomerular crescents.<sup>8–10</sup> The cells undergoing these changes include parietal epithelial cells,<sup>11–13</sup> renal progenitor cells,<sup>14</sup> infiltrating inflammatory cells,<sup>15</sup> and podocytes.<sup>16,17</sup> In addition, secondary tubulointerstitial damage arising in CGN also involves the proliferation of tubular epithelial cells (TECs) and myofibroblasts.<sup>18</sup> In previous studies, limitation of renal cell proliferation has been shown to attenuate renal injury in animal models,<sup>19,20</sup> and this strategy could be a more specific approach to prevent the development of maladaptive repair processes that lead to long-term renal scarring in CGN.

The mammalian target of rapamycin complex 1 (TORC1) protein kinase regulates intrinsic cell proliferation and growth, inflammation, and angiogenesis and is under investigation as a potential drug-target for the treatment of glomerular diseases.<sup>21,22</sup> In CGN, the efficacy of TORC1 inhibitors in induction therapy is not clear due to limited and conflicting preclinical data.<sup>23,24</sup> In a mouse model of anti-glomerular basement membrane (GBM) disease, the prophylactic administration of sirolimus (1 mg/kg/day by intraperitoneal injection) for 5 days prior to disease induction with heterologous nephritogenic antibody reduced subsequent glomerular injury.<sup>23</sup> In contrast, in rats with nephrotoxic serum nephritis (NSN), treatment with everolimus (2.5 mg/kg by oral gavage) at the same time as disease induction exacerbated glomerular fibrinoid necrosis but suppressed crescent formation.<sup>24</sup> The efficacy of TORC1 inhibitors on glomerular and tubulointerstitial injury following disease induction has also not been addressed. Therefore, in the present study, we tested the hypothesis that suppression of renal cell proliferation with the early administration of a TORC1 inhibitor attenuates subsequent glomerular and tubulointerstitial damage in rats with NSN.

## Materials and methods

### Animals

Adult male Wistar-Kyoto rats (6–8 weeks old, mean weight  $149 \pm 2$  g;  $n=12$ ) were obtained from the Animal Resources Centre (Perth, Australia) 7 days prior to commencement of the study and weighed (day 7). Rats were housed under standard conditions, with free access to food and water, and all

experimental procedures were approved by the Animal Ethics Committee, Westmead Hospital (Protocol number 4089).

### Animal model of CGN and experimental design

Sheep anti-rat nephrotoxic serum was prepared by immunizing sheep with a particulate fraction of GBM from normal rat kidney.<sup>25</sup> On day 0, rats received a single tail-vein injection of sheep anti-rat GBM serum (1 mL/kg;  $n=8$ ) under isoflurane anesthesia. On day 1, animals with NSN were divided into groups according to body weight and received either daily (between 7–8 am) subcutaneous injections of sirolimus (0.25 mg/kg, LC Laboratories, Woburn, MA, USA) or an equal volume of vehicle (20% dimethylsulfoxide, 20% ethanol, 60% saline v/v). A separate group of animals received a single injection of saline instead of nephrotoxic serum and were designated control animals ( $n=4$ ). Sirolimus (0.25 mg/mL in vehicle) was prepared under sterile conditions from stock solution on a daily basis prior to injection. The dose of sirolimus was chosen according to previous studies from our laboratory.<sup>26,27</sup> On day 14, all animals were anesthetized with excess ketamine and xylazine (90:10 mg/kg by intraperitoneal injection), blood was collected, and nephrectomies were performed via a midline laparotomy.

### Renal function

Rats were placed in metabolic cages for 14 hours on days 6 and day 13. Hematuria and leukocyturia were assessed by semiquantitative analysis of urinalysis reagent strips (Mutistix 8SG; Bayer, Pymble, Australia). Creatinine, protein, and albumin in serum were measured using an autoanalyzer (Roche Modular System; Hoffman-La Roche Ltd, Basel, Switzerland) at the Institute of Clinical Pathology and Medical Research, Westmead Hospital.

### Renal histology, immunohistochemistry, and immunofluorescent staining

Kidney tissue was fixed in either 10% neutral-buffered formalin or methyl carnoy solution and embedded in paraffin. Light microscopy was performed on 3  $\mu$ m sections stained with periodic acid–Schiff's reagent and hematoxylin. Immunohistochemistry was performed as previously described to detect proliferation, inflammation, and renal injury.<sup>27</sup> Briefly, a three layer immunoperoxidase staining system was used to detect 1) proliferating nuclei with an antibody against the nuclear antigen Ki-67 (Neomarkers Inc., Fremont, CA, USA); 2) monocytes/macrophages using a murine monoclonal immunoglobulin (Ig)G<sub>1</sub> antibody against

CD68 (ED-1) (1:200, AbDSerotec; Bio-Rad Laboratories, Hercules, CA, USA); 3) myofibroblast accumulation by a murine monoclonal IgG<sub>2</sub> antibody against  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (1:200, Sigma-Aldrich, St Louis, MO, USA); 4) TEC dedifferentiation using a murine monoclonal IgG antibody against vimentin (Dako, Glostrup, Denmark); and 5) activation of TORC1 signaling, determined by assessment of the downstream target using an antibody against phosphorylated S6 ribosomal protein (Ser 235/236)<sup>27</sup> (Cell Signaling, Danvers, MA, USA). Binding of the primary antibody was detected in sections using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen (brown color), with methyl green as the counterstain. In separate experiments, double-staining for Ki-67 and ED-1 was performed using two rounds of antibody: first with Ki-67 followed by the secondary antibody and DAB (positive cells have brown nuclei), and then with ED-1 followed by a secondary antibody and an alkaline phosphatase streptavidin (AP-red) chromogen (Zymed, San Francisco, CA, USA) (positive cells have red cytoplasm).

To assess the autologous antibody deposition in the glomerulus, frozen sections (6  $\mu$ m) of kidney were incubated with biotin conjugated anti-rat IgG antibody (AbDSerotec), followed by fluorescein isothiocyanate-conjugated streptavidin (BD Biosciences, San Jose, CA, USA), and then counterstained with 4',6-diamidino-2-phenylindole and Sudan black. Sections were then viewed with a confocal microscope.

## Quantitation of renal histology and immunohistochemistry

To assess glomerular injury, the percentage of glomeruli affected by fibrinoid necrosis or crescent formation on coronal sections was determined for each animal. In addition, images of glomeruli were digitized (Olympus DP11; Olympus Corporation, Tokyo, Japan), and the mean glomerular capillary tuft area and cell number were determined using area morphometric measurements (Image J Software; National Institutes of Health [NIH], Bethesda, MD, USA).<sup>28</sup> For quantitation of proliferation, the mean number of Ki-67-positive glomerular cells (total of 20 equatorial glomeruli, viewed at a magnification  $\times$ 600) or TECs (total of 20 consecutive fields, viewed at magnification  $\times$ 400 using a 0.02 mm<sup>2</sup> graticule) was calculated. The mean number of ED-1-positive glomerular cells was assessed in 20 glomeruli ( $\times$ 400), and periglomerular  $\alpha$ -SMA accumulation was assessed by a semiquantitative score (0 to 4, with 4 representing circumferential and intense staining). Tubulointerstitial injury was assessed by a

semiquantitative score (0 to 4), and interstitial ED-1, interstitial  $\alpha$ -SMA accumulation, and the number of vimentin-positive tubules determined in 20 cortical fields (magnification,  $\times$ 400). Immunostaining for phosphorylated S6 ribosomal protein was assessed in the glomerulus as the mean number of positive staining cells in 20 glomeruli and in TECs (proximal and distal) using a semiquantitative score (0 to 4).

## Assessment of phosphorylated (Ser 235/236) S6 ribosomal protein by Western blotting

Renal cortical tissue was homogenized in lysis buffer containing protease inhibitors (pH 7.4; 50 mM Tris-HCl, 150 mM NaCl, 1 mL EDTA, 1% NP-40, 0.01% sodium dodecyl sulfate, 0.5 mM dithiothreitol, 1  $\mu$ g/mL aprotinin, 0.5  $\mu$ g/mL leupeptin, 0.5  $\mu$ g/mL pepstatin A, 1 mM NaVO<sub>4</sub>, 1 mM NaF). Equal concentrations of homogenates were loaded onto a polyacrylamide minigel (4–12%, Nu-Page<sup>®</sup>, Thermo Fisher Scientific, Waltham, MA, USA) and transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences, Amersham, UK). Blots were blocked overnight at 4°C in 5% powdered skim milk in tris-buffered saline and incubated for 60 minutes at room temperature with a rabbit monoclonal antibody against phosphorylated S6 ribosomal protein antibody (Cell Signaling), followed by alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma-Aldrich). Bands were detected using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Sigma-Aldrich). Quantification of protein levels was determined by densitometry using image J software (NIH), normalized to  $\beta$ -actin.

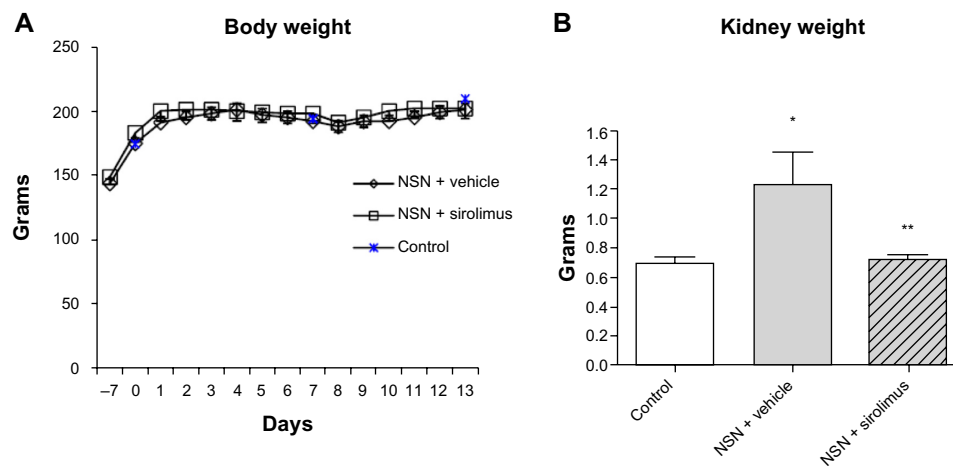
## Statistical analysis

Data were analyzed with JMP statistical software (SAS institute Inc., Cary, NC, USA) and expressed as mean  $\pm$  standard error of the mean. Comparisons between experimental groups were performed with the Kruskal–Wallis test followed by a post hoc analysis with the Tukey–Kramer HSD test. If the latter indicated a difference, the *P*-value was determined using the Wilcoxon test. A *P*-value of less than 0.05 indicated statistical significance.

## Results

### Effects of sirolimus on body weight, kidney enlargement, and renal function

There was no mortality or difference in body weight between the experimental groups (Figure 1A; Table 1). On day 14 in rats with NSN, treatment with sirolimus reduced the increase



**Figure 1** Effect of sirolimus on body weight and right kidney weight in rats with nephrotoxic serum nephritis (NSN).

**Notes:** (A) Time-course of body weight in the experimental groups during the study period. (B) Effect of sirolimus on right kidney weight in the experimental groups on day 14. Data are expressed as mean  $\pm$  standard error of the mean,  $n=4$  per group; \* $P<0.05$  compared to control; \*\* $P<0.05$  compared to NSN + vehicle.

in kidney enlargement by 41% (as determined by kidney weight and kidney to body weight ratio) and the decline in the endogenous creatinine clearance by 50% compared to the vehicle-treated group (all  $P<0.05$ ) (Figure 1B; Table 1). Sirolimus also reduced microscopic hematuria by 46% in NSN rats but did not affect the progression of the urine protein:creatinine ratio ( $P=0.27$ ) (Table 1).

**Table 1** Body weight, kidney weight, and parameters of renal function in the experimental groups

Parameter	Timepoint	Control	NSN + vehicle	NSN + sirolimus
BW (g)	Day 13	210 $\pm$ 7	201 $\pm$ 3	202 $\pm$ 3
KW (g)	Day 13	0.70 $\pm$ 0.05	1.23 $\pm$ 0.23*	0.72 $\pm$ 0.02**
KW:BW ratio (%)	Day 13	0.25 $\pm$ 0.01	0.61 $\pm$ 0.12*	0.36 $\pm$ 0.01**
Urine protein (mg/day)	Day 6	–	225 $\pm$ 34	98 $\pm$ 52
Urine p:cr ratio (mg/mmol)	Day 6	–	9.9 $\pm$ 1.4*	4.3 $\pm$ 2.4
Leukocyturia	Day 6	–	3.0 $\pm$ 0	1.4 $\pm$ 0.7
Hematuria	Day 6	–	2.0 $\pm$ 0.7	1.0 $\pm$ 0.6
Serum albumin (g/dL)	Day 13	34 $\pm$ 3	27 $\pm$ 1*	30 $\pm$ 2
Serum Cr ( $\mu$ mol/L)	Day 13	34 $\pm$ 4	53 $\pm$ 4*	44 $\pm$ 6
CrCl (mL/min)	Day 13	1.19 $\pm$ 0.65	0.38 $\pm$ 0.02*	0.57 $\pm$ 0.06**
CrCl (mL/min/g KW)	Day 13	1.60 $\pm$ 0.40	0.33 $\pm$ 0.05*	0.79 $\pm$ 0.08**

**Notes:** Data expressed as mean  $\pm$  standard error of the mean; \* $P<0.05$  compared to control; \*\* $P<0.05$  compared to NSN + vehicle.

**Abbreviations:** BW, body weight; Cr, creatinine; CrCl, endogenous creatinine clearance; KW, kidney weight; NSN, nephrotoxic serum nephritis; p:cr, protein to creatinine.

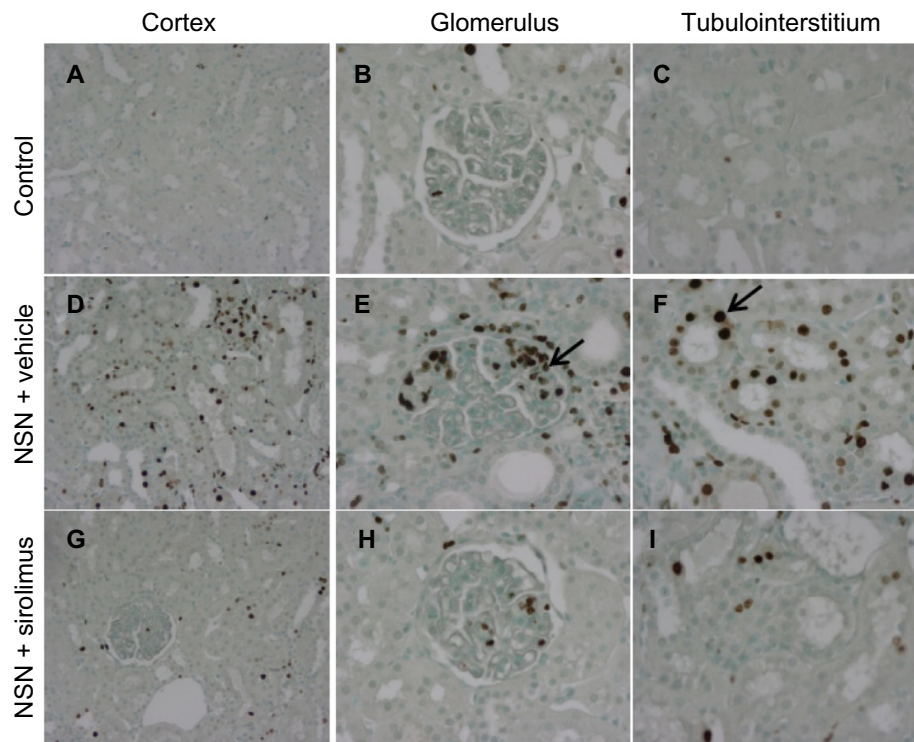
## Effects of sirolimus on renal cell proliferation

Glomerular cell proliferation (as determined by Ki-67 immunostaining) was increased 7.1-fold in the NSN + vehicle group compared to control (Figure 2; Table 2). Treatment with sirolimus in NSN reduced glomerular cell proliferation by 53% ( $P<0.05$ ), but this was still elevated compared to the control group ( $P=0.03$ ). In TECs, proliferation was increased 6.4-fold in the NSN + vehicle group compared to control, and this elevation was attenuated by 70% in the NSN + sirolimus group. In contrast to glomerular cell proliferation, the difference in the number of Ki-67 + TECs between the control and NSN + sirolimus groups did not reach statistical significance ( $P=0.15$ ).

## Effects of sirolimus on glomerular injury

Vehicle-treated rats with NSN developed a focal necrotizing glomerular injury characterized by glomerular fibrinoid necrosis, hypercellularity, and occasional crescent formation, with linear glomerular deposition of rat IgG (Figure 3). Following treatment with sirolimus in NSN, there was a significant reduction in glomerular hypercellularity and capillary tuft enlargement, glomerular fibrinoid necrosis, and the periglomerular deposition of  $\alpha$ -SMA (Table 2). This was associated with an abrogation of glomerular rat IgG deposition (Figure 3). However, treatment with sirolimus had no effect on glomerular crescent formation ( $P=0.15$ ) (Table 2) nor glomerular ED1-positive cell accumulation ( $P=0.25$ ) compared to the NSN + vehicle group (Table 2).





**Figure 2** Effect of sirolimus on renal cell proliferation (Ki-67-positive cells) in rats with nephrotoxic serum nephritis.

**Notes:** Representative photomicrographs showing immunohistochemistry for Ki-67 in the renal cortex ( $\times 100$ ) (panels **A**, **D** and **G**), glomeruli ( $\times 400$ ) (panels **B**, **E** and **H**), and the tubulointerstitium ( $\times 200$ ) (panels **C**, **F** and **I**) in the experimental groups (control, NSN + vehicle, and NSN + sirolimus) on day 14. Cells positive for Ki-67 are revealed by DAB-positive (dark brown) nuclear staining (arrows). Sections were counterstained with methyl green.

**Abbreviations:** DAB, 3,3'-diaminobenzidine tetrahydrochloride; NSN, nephrotoxic serum nephritis.

**Table 2** Quantitative assessment of glomerular injury and phosphorylated S6 immunostaining in the experimental groups

Parameter	Control	NSN + vehicle	NSN + sirolimus
Number of Ki-67-positive cells per GCS	0.8 $\pm$ 0.3	5.7 $\pm$ 0.8*	2.7 $\pm$ 0.6**
% glomeruli with fibrinoid necrosis	–	19.4 $\pm$ 3.1	6.1 $\pm$ 1.3**
% glomeruli with crescent formation	–	6.5 $\pm$ 2.2	3.4 $\pm$ 1.6
Glomerular capillary tuft area	1.0 $\pm$ 0.05	1.26 $\pm$ 0.01*	0.96 $\pm$ 0.04**
Number of nuclei per GCS	60.2 $\pm$ 0.5	81.1 $\pm$ 4.3*	66.5 $\pm$ 3.2**
Number of ED-1-positive cells per GCS	2.0 $\pm$ 0.2	10.4 $\pm$ 2.1*	6.1 $\pm$ 2.1
Number of ED-1+/Ki-67+ cells per GCS	0 $\pm$ 0	0.8 $\pm$ 0.3	0.5 $\pm$ 0.3
Ki-67+/ED-1+ cells divided by total Ki-67+ cells (%)	0 $\pm$ 0	14.6 $\pm$ 5.9	11.1 $\pm$ 2.7
Ki-67+/ED-1+ cells divided by total ED-1+ cells (%)	0 $\pm$ 0	56.2 $\pm$ 11.9	36.1 $\pm$ 14.7
Periglomerular $\alpha$ -SMA accumulation	0.7 $\pm$ 0.3	3.0 $\pm$ 0.5*	1.2 $\pm$ 0.3**
Glomerular staining for phosphorylated S6 (Ser 235/236)	14.8 $\pm$ 1.2	18.1 $\pm$ 1.2	6.6 $\pm$ 1.3**

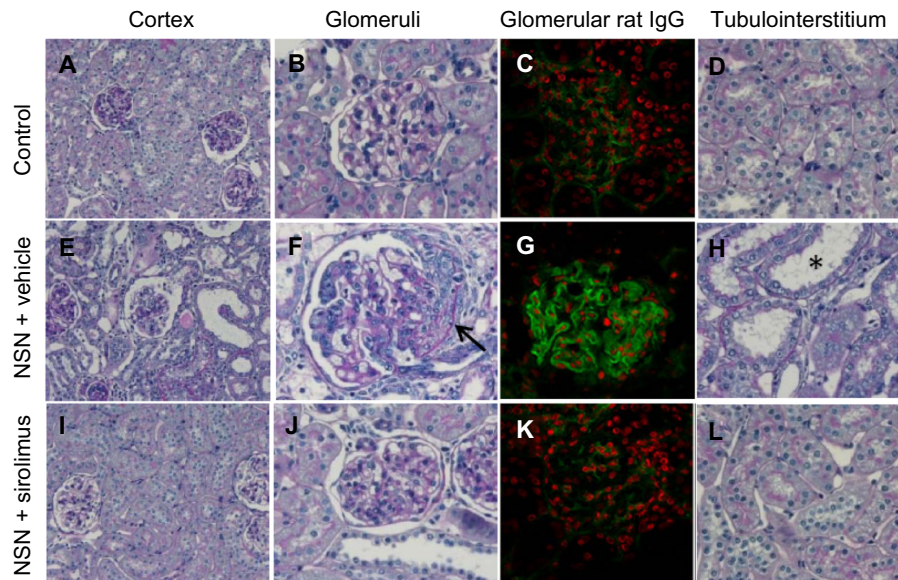
**Notes:** Data expressed as mean  $\pm$  standard error of the mean; \* $P < 0.05$  compared to control; \*\* $P < 0.05$  compared to NSN + vehicle.

**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; GCS, glomerular cross section; NSN, nephrotoxic serum nephritis.

Double-labelling experiments were performed to determine whether sirolimus altered the number of proliferating monocytes/macrophages in the glomerulus. In vehicle-treated NSN rats, ED1-positive cells represented 14.6% of the total number of proliferating (Ki-67-positive) cells, and this was not altered by sirolimus (Table 2). In addition, 56.2% of ED-1 positive cells were proliferating (Ki-67 positive), but neither the proportion nor the mean number of double-labelled cells per glomerulus was altered by sirolimus (Table 2).

### Effects of sirolimus on tubulointerstitial damage

NSN was accompanied by severe and diffuse tubulointerstitial damage, consisting of tubular dilatation, TEC dedifferentiation, loss of proximal TEC brush border, and interstitial inflammation and fibrosis (Figure 3). Treatment with sirolimus markedly prevented the development of tubulointerstitial damage in rats NSN, such that the light microscopic appearance was almost similar to the control rats (Figure 3; Table 3). This was associated with significant reductions in interstitial ED-1- and  $\alpha$ -SMA-positive cell accumulation as well as in the tubular expression of vimentin (Table 3). Sirolimus also reduced the number of cells double-labelled with ED-1



**Figure 3** Effect of sirolimus on renal histology and glomerular rat IgG deposition in rats with nephrotoxic serum nephritis.

**Notes:** Representative photomicrographs showing renal histology and immunofluorescence staining in the glomerulus for rat IgG deposition in the experimental groups on day 14. Renal histology is shown in periodic-acid Schiff-stained sections of cortex ( $\times 100$ ) (panels **A**, **E** and **I**), glomerulus ( $\times 400$ ) (panels **B**, **F** and **J**) and tubulointerstitium ( $\times 200$ ) (panels **D**, **H** and **L**). Glomerular rat IgG was detected by immunofluorescence using confocal microscopy ( $\times 400$ ) (panels **C**, **G** and **K**). Rats with NSN + vehicle developed glomerular fibrinoid necrosis and occasional crescent formation (arrow in **F**) with linear glomerular deposition of rat IgG deposition. The glomerular changes were accompanied by severe and diffuse tubulointerstitial damage, consisting of tubular dilatation, TEC dedifferentiation, and loss of proximal TEC brush border (asterisk in **H**).

**Abbreviations:** IgG, immunoglobulin G; TEC, tubular epithelial cell; NSN, nephrotoxic serum nephritis.

and Ki-67 by 89%, indicating that interstitial monocyte proliferation was also attenuated (Table 3).

### Effect of sirolimus on the renal expression of phosphorylated (Ser 235/236) S6 ribosomal protein

By Western blot analysis, sirolimus attenuated the increase in renal cortical expression of phosphorylated S6 protein

**Table 3** Quantitative assessment of tubulointerstitial injury and phosphorylated S6 immunostaining in the experimental groups

Parameter	Control	NSN + vehicle	NSN + sirolimus
Number of Ki-67-positive cells ( $\text{mm}^2$ )	43.3 $\pm$ 8.5	276.2 $\pm$ 16.6*	84.2 $\pm$ 30.5**
Cortical tubulointerstitial injury score	0 $\pm$ 0	3.4 $\pm$ 0.4*	1.4 $\pm$ 0.7**
Interstitial ED-1-positive cells ( $\text{mm}^2$ )	3.1 $\pm$ 0.9	31.8 $\pm$ 9.7*	3.5 $\pm$ 0.7**
Interstitial ED-1+/Ki-67+ cells	0 $\pm$ 0	2.0 $\pm$ 0.9*	0.6 $\pm$ 0.3**
Tubulointerstitial vimentin	0.4 $\pm$ 0.1	2.5 $\pm$ 0.4*	1.4 $\pm$ 0.3**
Interstitial $\alpha$ -SMA accumulation	0.3 $\pm$ 0.1	3.1 $\pm$ 0.2*	1.7 $\pm$ 0.9**
Tubulointerstitial staining for p-S6 (Ser 235/236)	1.2 $\pm$ 0.3	2.9 $\pm$ 0.3*	1.0 $\pm$ 0.5**

**Notes:** Data expressed as mean  $\pm$  standard error of the mean; \* $P < 0.05$  compared to control; \*\* $P < 0.05$  compared to NSN + vehicle.

**Abbreviations:**  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; NSN, nephrotoxic serum nephritis; p-S6, phosphorylated S6 ribosomal protein.

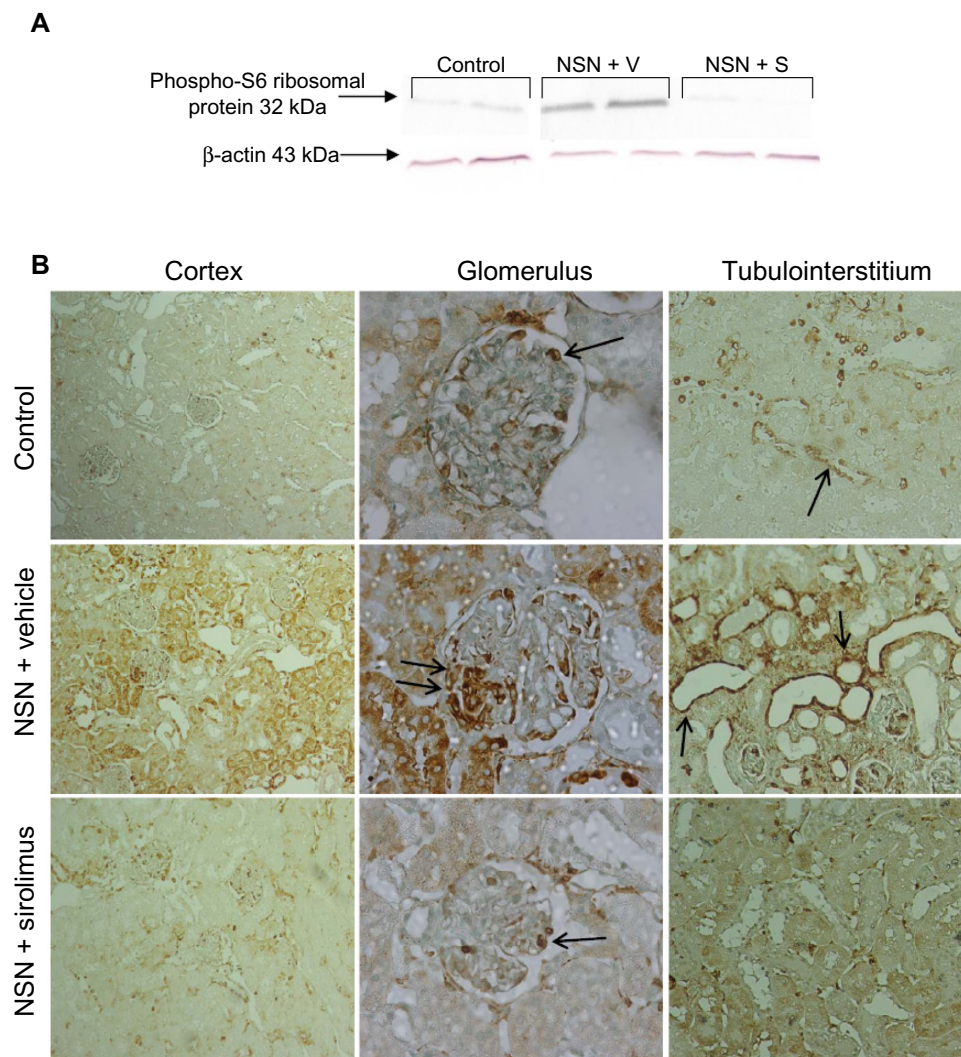
in rats with NSN (Figure 4A). By immunohistochemistry, the expression of phosphorylated S6 ribosomal protein was increased in glomerular and TECs in NSN + vehicle group compared to control rats, and significantly attenuated with sirolimus treatment (Figure 4B; Tables 2 and 3).

### Discussion

The results of this study demonstrate that the early administration of sirolimus has protective effects on subsequent renal injury in rats with NSN. Specifically, treatment with sirolimus attenuated renal cell proliferation, glomerular fibrinoid necrosis and improved tubulointerstitial injury despite the persistence of heavy proteinuria. The new findings in the current study were that 1) the beneficial effects were present even if sirolimus was commenced 1 day after injection with anti-GBM serum and 2) the improvement in renal injury was more consistent in the tubulointerstitium than the glomerular compartment. These results extend previous data showing that the prophylactic administration of sirolimus 5 days prior to disease induction reduced renal injury in a mouse model of anti-GBM disease<sup>23</sup> but conflict with the finding that everolimus exacerbated glomerular fibrinoid necrosis in a rat model of NSN.<sup>24</sup>

TORC1 is a critical component of the intracellular mitogenic protein kinase pathway (see Figure 5) that regulates intrinsic renal cell proliferation and hypertrophy





**Figure 4** Effect of sirolimus (S) on the renal expression of phosphorylated S6 (phospho-S6) ribosomal protein (Ser 235/236) in rats with nephrotoxic serum nephritis.

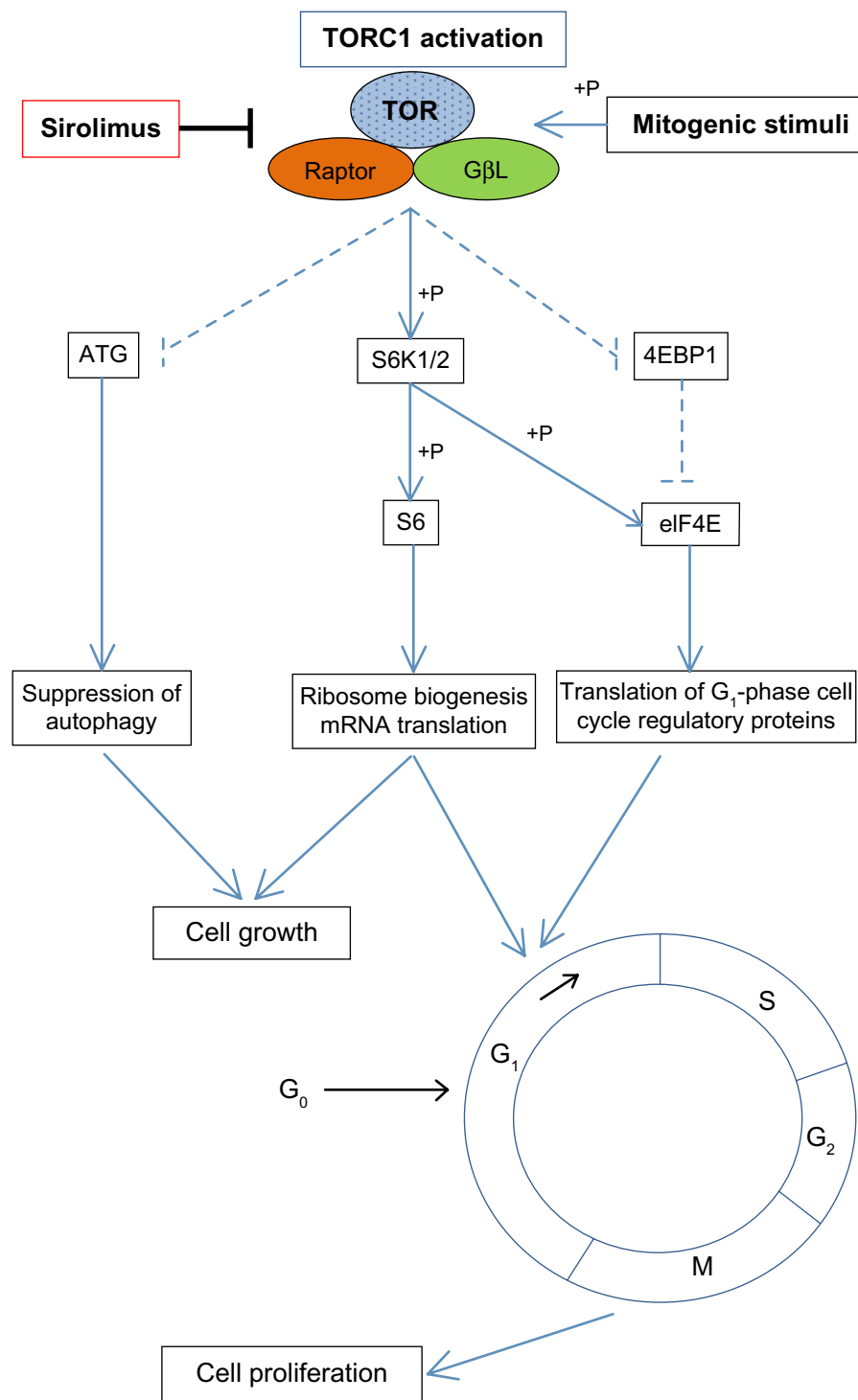
**Notes:** (A) Western blot showing the expression of phosphorylated S6 ribosomal protein in renal cortical homogenates; (B) Representative photomicrographs showing immunohistochemistry for phosphorylated S6 ribosomal protein in the cortex ( $\times 100$ ), glomerulus ( $\times 400$ ), and tubulointerstitium ( $\times 200$ ). Cells positive for S6 are revealed by DAB-positive (dark brown) cytoplasmic staining (arrows); sections were counterstained with methyl green.

**Abbreviations:** DAB, 3,3'-diaminobenzidine tetrahydrochloride; NSN, nephrotoxic serum nephritis; V, vehicle.

in a variety of kidney disease models, including diabetic nephropathy,<sup>29</sup> mesangioliferative glomerulonephritis,<sup>30</sup> membranous nephropathy,<sup>31</sup> chronic ureteral obstruction,<sup>32</sup> renal ischemia,<sup>33</sup> and polycystic kidney disease.<sup>34</sup> Consistent with these observations and not surprisingly, in the present study, sirolimus markedly reduced both glomerular and TEC proliferation as assessed by the nuclear antigen Ki-67. The reduction in cell proliferation was also coupled with a significant decrease in the whole renal expression of phosphorylated S6 ribosomal protein, confirming inhibition of the TORC1 signaling pathway with sirolimus treatment. Interestingly, the reduction in NSN-induced glomerular cell proliferation with sirolimus was only partial, and it remained significantly elevated (3.3-fold) compared to the control group. This

incomplete effect on proliferation in the glomerulus could be due to cell-specific effects of TORC inhibition on specific intrinsic glomerular cells.

Glomerular fibrinoid necrosis is a characteristic feature of CGN and a consequence of severe cell-mediated and/or immune-mediated injury in response to immune-complex or antibody deposition.<sup>1,2</sup> Treatment with sirolimus reduced glomerular fibrinoid necrosis by 69%. However, differences in glomerular crescent formation, glomerular monocyte, or in proteinuria between the two disease groups did not reach statistical significance. Moreover, treatment with sirolimus did not alter glomerular monocyte/macrophage proliferation, suggesting that 1) macrophage proliferation in the glomerulus is insensitive to TORC1 and/or 2) the accumulation of glomerular



**Figure 5** Molecular mechanisms by which sirolimus inhibits cell growth and proliferation.

**Notes:** The target of rapamycin complex I (TORC1) is a key regulator of mitogenesis in mammalian cells. In the cytosol, TORC1 exists as a complex consisting of TOR, raptor and GβL. Phosphorylation (+P) of TORC1 by upstream signal transduction pathways (not shown), in response to mitogenic and other stimuli, leads to cell proliferation and growth, primarily through the activation of two key downstream substrates that control protein synthesis/ribosome biogenesis and cap-dependent translation: 1) the ribosomal S6 kinase (S6K) family and 2) the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs). Activation of p70 S6 kinase and subsequent phosphorylation of S6 ribosomal protein leads to the translation of ribosome proteins and components of the translational machinery. In parallel, the phosphorylation 4E-BP1 releases it from the translation factor, eIF-4E, and allows the latter to initiate the translation of cyclin D1 and D3 messenger RNA (mRNA). Both of these events mediate G<sub>1</sub>-S phase cell cycle progression. TORC1 is also a negative regulator of autophagy via autophagy-related proteins (ATG), and this regulates cell growth. In the cytosol, sirolimus binds to FK506-binding protein-12 (not shown), and this complex is a highly specific and irreversible allosteric inhibitor of the kinase activities of TORC1, and thus causes G<sub>1</sub>-phase cell cycle arrest and suppression of proliferation. In some cell types, such as podocytes, prolonged exposure to sirolimus also inhibits TORC2 and promotes apoptosis via Akt2 (not shown).



macrophages at day 14 is due to other mechanisms, such as chemotactic signals from intrinsic renal cells in the glomerulus in response to ongoing immune-mediated injury.<sup>15</sup>

In contrast, the tubulointerstitium in sirolimus-treated NSN rats appeared pristine, with the exception of proteinaceous material being present in tubular lumens. In addition, all immunohistochemical markers of tubulointerstitial injury were consistently attenuated by sirolimus (including interstitial monocyte/macrophage and myofibroblast accumulation, the number of vimentin-positive tubules, interstitial monocyte/macrophage proliferation). This improvement was observed despite the presence of ongoing heavy proteinuria, supporting the hypothesis that sirolimus suppresses the plasma-protein-induced TEC inflammatory response.<sup>31</sup>

However, previous data show that the timing and dose of TORC1 inhibitors in CGN appear to be a critical determinant of whether it is protective or detrimental. Specifically, preclinical studies demonstrate that renal injury is worsened if TORC1 inhibitors are administered in late-stage disease, when chronic kidney disease has become established.<sup>23,24,35</sup> This double-edged paradigm may be because TORC1 signaling promotes glomerular capillary repair processes via activation of vascular endothelial cell growth factor.<sup>36</sup> In addition, prolonged treatment with sirolimus inhibits both TORC1 and TORC2,<sup>37</sup> and accumulating data indicate that TORC2 inhibition promotes podocyte apoptosis and foot process effacement via Akt2.<sup>38,39</sup> Furthermore, high-doses of TORC1 inhibitors caused de novo crescent formation in a mesangioproliferative rat model of glomerulonephritis.<sup>36</sup> This then raises the question as to whether the absence of complete protection in glomerular injury and proteinuria in the current study is, in fact, reflective of the foregoing mechanisms. Alternatively, the persistence of proteinuria could also be a consequence of starting treatment after the onset of initial glomerular injury (and therefore, the incomplete protection to the glomerulus could also be potentially shared by other immunosuppressive drugs). Although the dual roles of TORC1 in kidney disease could limit the therapeutic utility of currently available TORC1 inhibitors in the treatment of immune-mediated CGN in humans, further carefully undertaken preclinical studies using additional models are needed. In this regard, the efficacy of sirolimus could also be dependent on the immunopathogenesis of the underlying etiology of CGN (Table 4).<sup>40,41</sup>

In conclusion, this study provides novel preclinical evidence that induction treatment with sirolimus has selected protective effects in a crescentic model of glomerulonephritis, supporting the hypothesis that the TORC1 pathway is a

**Table 4** Classification and etiology of CGN

Immunopathologic category	Etiology	% of CGN cases
Linear glomerular IgG IF staining + circulating anti-GBM antibodies	With lung hemorrhage: Goodpasture syndrome Without lung hemorrhage: anti-GBM disease	~15%
Granular glomerular IF staining with immune complex localization on EM; Serology varied and dependent on cause	IgA nephropathy and Henoch–Schönlein purpura Lupus nephritis Acute postinfectious glomerulonephritis Other glomerular diseases (membranoproliferative, membranous, fibrillary)	~24%
Paucity of glomerular IF immunoglobulin staining + circulating ANCA	ANCA glomerulonephritis Microscopic polyangiitis Granulomatosis with polyangiitis (Wegener's) Eosinophilic granulomatosis with polyangiitis (Churg–Strauss)	~60%
Other		~1%

**Note:** Adapted from *Brenner and Rector's The Kidney*, Vol 1. 9th ed, Nachman PH, Jennette C, Falk RJ, Primary glomerular disease, 1100–1191, Copyright © Elsevier (2012).<sup>41</sup>

**Abbreviations:** ANCA, antineutrophil cytoplasmic antibody; CGN, crescentic glomerulonephritis; EM, electron microscopy; GBM, glomerular basement membrane; IF, immunofluorescence; IgA, immunoglobulin A; IgG, immunoglobulin G.

mediator, in part, in this pattern of glomerular injury. Further preclinical studies should compare the efficacy of sirolimus with conventional (cyclophosphamide and corticosteroids)<sup>42</sup> and novel agents<sup>43</sup> as well as drugs targeting fibrinolytic pathways.<sup>44</sup> The cell-specific mechanisms underlying the compartment-specificity of TORC1 inhibition in CGN also requires further elucidation.

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