Spleen tyrosine kinase promotes acute neutrophil-mediated glomerular injury via activation of JNK and p38 MAPK in rat nephrotoxic serum nephritis

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Glomerular antibody deposition induces acute neutrophil-mediated glomerular injury via activation of c-Jun amino terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). However, the link between antibody deposition and activation of JNK/p38 MAPK signalling is unclear. This study tested the postulate that spleen tyrosine kinase (Syk), which is activated via Fcγ-receptor ligation, is required for activation of JNK and p38 signalling and acute neutrophil-mediated glomerular injury. We used a Syk inhibitor (SYKi) in rat nephrotoxic serum nephritis (NTN) in which neutrophil-mediated glomerular injury is dependent upon JNK and p38 signalling. SYKi or vehicle treatment of Sprague–Dawley rats began 30 min before administration of anti-GBM serum with rats killed 3 or 24 h later. Immunostaining identified *de novo* glomerular Syk activation (p-Tyr 525/526) in untreated NTN, being most prominent in neutrophils. Vehicle and untreated NTN exhibited heavy proteinuria and glomerular thrombosis at 24 h with P-selectin and fibrin immunostaining within capillaries, glomerular macrophage and T cell infiltration, activation of JNK and p38 MAPK signalling, and upregulation of glomerular mRNA levels of pro-inflammatory molecules (TNF-α, NOS2, MMP-12 and CCL2). In contrast, SYKi treatment provided complete protection from proteinuria, with a profound reduction in glomerular thrombosis and immunostaining for P-selectin and fibrin, and a substantial reduction in glomerular mRNA levels of pro-inflammatory molecules. SYKi treatment also reduced the acute glomerular neutrophil influx and pro-inflammatory response at 3 h in NTN. These protective effects were associated with a significant reduction in glomerular JNK and p38 MAPK activation. In addition, activation of Syk, JNK and p38 was identified in human biopsy samples of acute crescentic glomerulonephritis. In conclusion, this study demonstrates that Syk signalling is required for JNK and p38 MAPK signalling and acute neutrophil-dependent glomerular injury in rat NTN. These findings identify Syk as a potential therapeutic target in antibody-dependent kidney disease.

Studies of experimental kidney disease have established that glomerular antibody deposition can induce acute podocyte injury and proteinuria through Fc-receptor-dependent recruitment and activation of circulating neutrophils and monocytes.[1–3] Leukocyte-mediated renal injury in antibody-dependent models of kidney disease, such as nephrotoxic serum nephritis (NTN), operates via activation of the stress-activated protein kinases, c-Jun amino terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK).[4,5] However, we do not know the upstream components involved in activation of JNK and p38 MAPK pathways in this type of glomerular injury. One potential mechanism of JNK/p38 activation in antibody-dependent glomerular injury is through Fc-receptor activation.

Spleen tyrosine kinase (Syk) is a 72-kDa non-receptor tyrosine kinase that has an important role in receptor signalling in cells of the haemotopoeitic system including B cells, neutrophils and monocyte/macrophages, but is largely absent in T cells.[6] Syk is best known as a component of signalling via B cell receptors and Fc-receptors.[7–10] In these, a ligand-stimulated receptor associates with a transmembrane protein that contains an immunoreceptor tyrosine-based activation motif, which is then rapidly phosphorylated by SRC family kinases, leading to recruitment and activation of

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Syk. Syk is activated by phosphorylation at Tyr 525/526 and then binds to members of the VAV and phospholipase Cγ families, which in turn lead to activation of several signalling pathways, including JNK and p38 MAPK.[11]

Syk inhibitor drugs have produced positive results in clinical trials of rheumatoid arthritis and seasonal rhinitis.[12–15] Preclinical studies using Syk inhibitor drugs have provided evidence that blockade of Syk protects against antibody-dependent kidney disease murine lupus.[16,17] In addition, a Syk inhibitor has been shown to suppress macrophage-mediated renal injury in a model of NTN in WKY rats, which lacks the heterologous phase of neutrophil-dependent glomerular injury.[18] Despite these promising results, however, we have no information regarding Syk activation in kidney disease. Thus, three important questions remain unresolved. First, is Syk activated in antibody-dependent glomerular injury? Second, is Syk activation required for activation of JNK/p38 MAPK signalling in antibody-dependent glomerular injury? Third, is Syk activation required for antibody-dependent acute neutrophil-mediated glomerular injury? To address these questions, we used a Syk inhibitor (SYKi) in a model of NTN in Sprague–Dawley rats in which proteinuria in the first 24 h is neutrophil mediated.[19]

MATERIALS AND METHODS

Syk Inhibitor
The SYKi was synthesised by Celgene. SYKi inhibits Syk enzyme activity with an IC_{50} of 30 nM and has partial activity against Flt3 (66 nM), KDR (107 nM) and JAK2 (116 nM); but has no activity against a panel of 55 other kinases including p38, JNK, c-kit ($>3\,\mu$M), Lyn or Lck ($\sim1\,\mu$M). This is similar to the specificity of the most widely reported Syk inhibitor, R406.[20] SYKi inhibits IgM-induced TNF-α production by human macrophages and anti-IgM activation of human B cells (data not shown).

Rat Model of NTN
All animal experiments were approved by Monash Medical Centre Animal Ethics Committee. NTN was induced in inbred female Sprague–Dawley rats (150–200 g; Monash Animal Services, Melbourne) as described previously.[4,19] Briefly, rats were immunised by subcutaneous injection of sheep IgG in Freund's complete adjuvant followed 5 days later (termed day 0) by intravenous injection of sheep anti-rat GBM serum. Animals were killed 3 or 24 h after administration of anti-GBM serum injection. SYKi was prepared
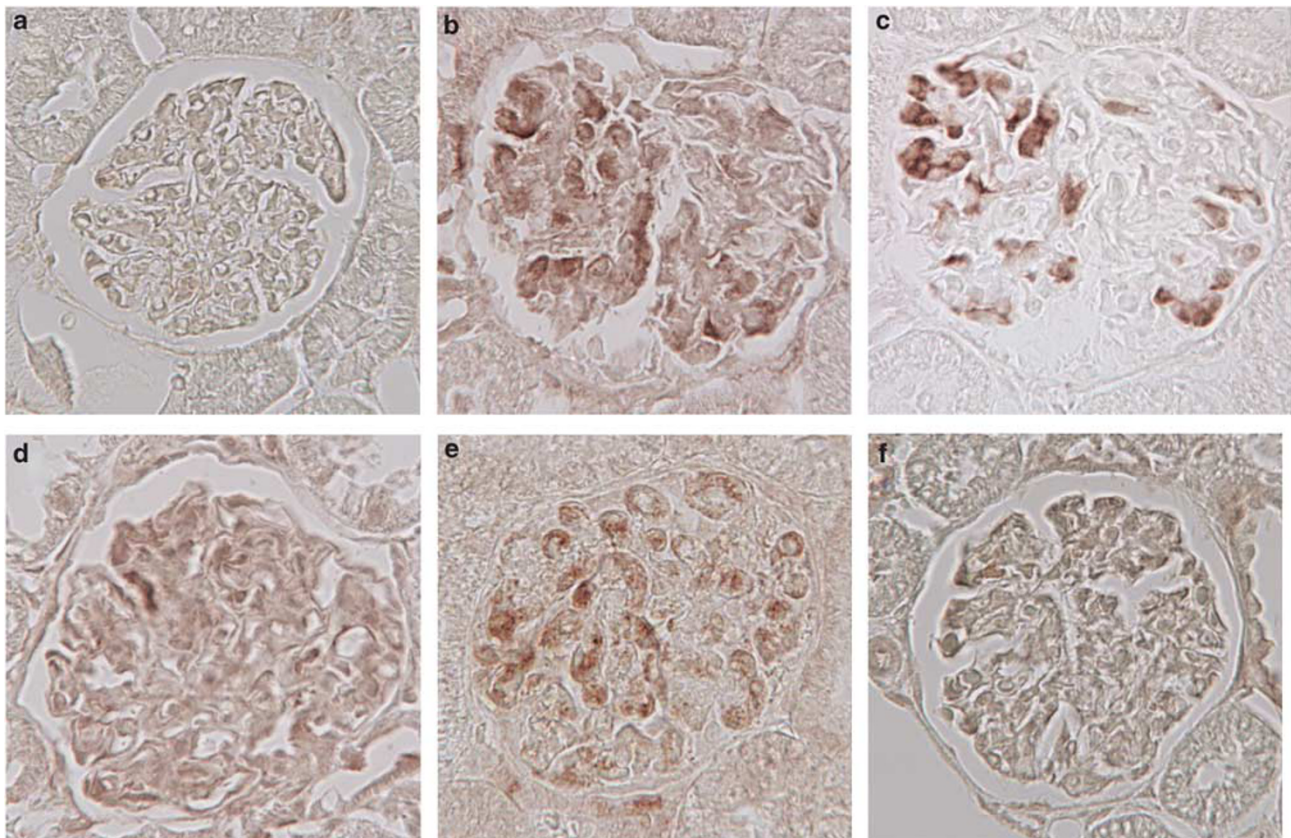

Figure 1 Immunostaining for p-Syk (Tyr 525/526) and RP-1 positive neutrophils in rat NTN. (**a**) Normal rat kidney lacks p-Syk staining. (**b**) Focal staining pattern of p-Syk staining in capillary loops in 3-h untreated NTN, which co-localises with (**c**) infiltrating RP1 + neutrophils. (**d**) In contrast, p-Syk staining is markedly reduced in NTN with SYKi treatment. (**e**) p-Syk staining is seen in capillary loops at 24 h in untreated NTN, and (**f**) this is abolished with SYKi treatment.

as a suspension in 10% 2-hydropropyl-β-cyclodextrin (vehicle) and given by intraperitoneal injection, 10 mg/kg, every 6 h.

In the first experiment, groups of six or seven animals with SYKi treatment, vehicle treatment or no treatment were killed 24 h after administration of anti-GBM serum. SYKi treatment began 30 min before anti-GBM serum injection and continued until animals were killed at 24 h. In addition, a group of normal rats was examined. Animals were housed in metabolic cages for 23 h after anti-GBM serum to collect urine. In the second experiment, groups of six animals were given SYKi or vehicle treatment 30 min before anti-GBM serum injection and animals were killed at 3 h.

Blood was collected from animals at the time of death and white blood cell counts were performed on a Cell Dyn 3500 Cell Counter. Tissues from one kidney were either snap frozen, or fixed in 2% paraformaldehyde or 4% formalin and processed for histopathological analysis. One kidney from the animals in each group ($n = 4$–6) was used for glomerular isolation by differential sieving, with tubular fragments removed under microscopic examination. Urinary protein excretion was estimated using a Coomassie (Bradford) Protein Assay Kit (ThermoScientific, Rockford, USA).

Renal Biopsies
Formalin-fixed sections of kidney biopsy tissue was analysed from patients who had previously undergone diagnostic renal biopsy at Monash Medical Centre. Patients gave informed consent and this study was approved by the Monash Medical Centre Human Ethics and Research Committee. The four biopsies were of minimal change disease, thin membrane disease and two cases of post-infectious glomerulonephritis. None of the patients were on immunosuppressive therapy at the time of biopsy.

Antibodies
The following antibodies were used in this study: mouse anti-rat CD68 recognising monocytes/macrophages (ED1) and mouse anti-rat T-cell receptor (R73, Serotec, Oxford, UK); mouse anti-rat neutrophil (RP-1, Becton Dickinson, San Diego, CA, USA); rabbit anti-fibrinogen-γ, mouse anti-P-selectin (CD62P) and rabbit anti-WT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit antibodies against phospho-Syk (Tyr 525/526), phospho-p38 (Thr 180/Thr 182), phospho-JNK (Tyr183/Tyr185) (Cell Signalling, Danvers, MA, USA), rabbit anti-human neutrophil elastase (Abcam, Cambridge, MA) and; rabbit anti-tubulin (Abcam). Biotinylated secondary antibodies were goat anti-mouse IgG (Zymed, San Franciso, CA, USA) and goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), which were detected using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Other secondary detection systems included goat anti-rabbit Alexa Fluor 680 (Molecular Probes, Eugene, OR, USA), horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG and mouse peroxidise-conjugated anti-peroxidase complexes (PAP) (Dako, Glostrup, Denmark).

Histology
Periodic acid-Schiff staining was performed on 2 μm sections of formalin-fixed tissue sections. A total of 30 glomeruli from each animal were examined under high power ($\times 400$). The area of the glomerular tuft affected by capillary thrombi was assessed using a semi-quantitative score: 0 (Nil), 1 ($<25\%$), 2 (25 to 50%), 3 (50 to 75%) and 4 ($>75\%$). Analysis was performed on blinded slides.

Immunohistochemistry
Immunoperoxidase staining for fibrinogen-γ and WT-1 was performed on 4-μm sections of formalin-fixed tissue sections using microwave-based antigen retrieval (citrate buffer, pH 6.0 or Tris-HCL, pH 8.5, respectively) followed by the Vectastain ABC method as previously described.[21] Immunostaining for p-Syk, p-p38, p-JNK, CD68 and human neutrophils was performed on formalin-fixed sections with antigen retrieval (citrate buffer, pH 6.0). Immunostaining for rat neutrophils, T cells and P-selectin was performed on frozen sections using the PAP or ABC method.

The number of glomerular cells stained positive for RP1, R73, ED1, WT-1 and p-JNK were counted in at least 30 glomerular cross-sections from each animal under high power ($\times 400$). Glomerular immunostaining for P-selectin, fibrinogen and p-p38 was assessed using a semi-quantitative score: 0 (Nil), 1 ($<25\%$), 2 (25 to 50%), 3 (50 to 75%) and 4 ($>75\%$). All analysis was performed on blinded slides.

Real-Time PCR
Real-time PCR was performed as described previously.[22] RNA was extracted from isolated glomeruli and reverse transcribed using random primers to make cDNA (First-
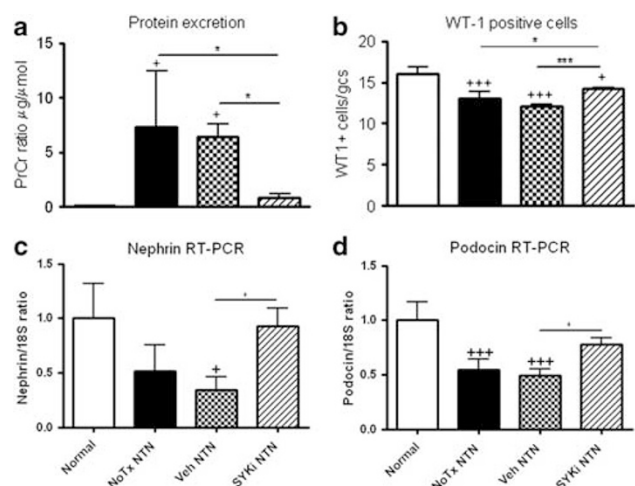

Figure 2 Renal injury in NTN. (**a**) Graph of urinary protein to creatinine ratio (PrCr in μg/μmol). (**b**) Graph showing the number of WT-1 + podocytes per glomerular cross-section (gcs). Real-time RT-PCR of isolated glomeruli showing relative mRNA levels for nephrin (**c**) and (**d**) podocin. $^+P < 0.05$, $^{+++}P < 0.001$ vs normal, $^*P < 0.05$, $^{***}P < 0.001$ vs SYKi.

Strand Synthesis System for RT-PCR, Invitrogen). Real-time PCR was performed on a StepOne machine (Applied Biosystems, Scoresby, VIC, Australia) with thermal cycling conditions of 37 °C for 10 min, 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s, 60 °C for 20 s and 68 °C for 20 s. The primer pairs and FAM-labeled MGB probes were as
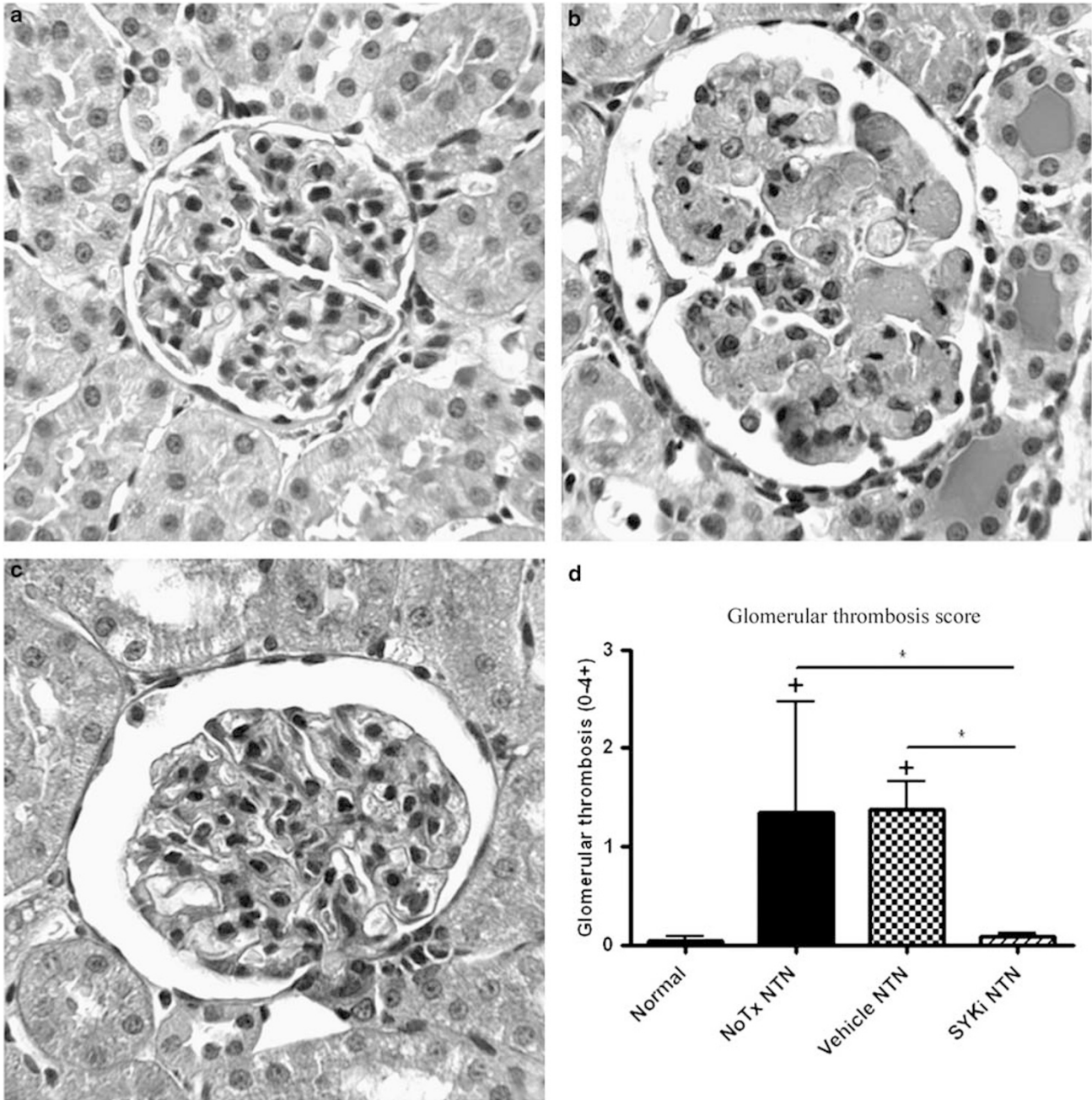

Figure 3 Renal damage assessed on PAS-stained sections. (**a**) Normal rat kidney. (**b**) Untreated 24-h NTN showing glomerular capillary thrombosis and tubular protein casts. (**c**) A 24 h NTN with SYKi treatment showing mild hypercellularity, but no thrombi. (**d**) Graph of glomerular thrombosis score (0–4 +). [+]$P < 0.05$ *vs* normal, *$P < 0.05$ *vs* SYKi.

Figure 4 Immunostaining for P-selectin and fibrinogen in rat NTN. Immunostaining for P-selectin in (**a**) normal rat kidney, (**b**) untreated 24-h NTN and (**c**) SYKi-treated 24-h NTN. (**d**) Graph showing semi-quantitative scoring of glomerular P-selectin staining. Immunostaining for fibrin deposition in (**e**) normal rat kidney, (**f**) untreated 24-h NTN and (**g**) SYKi-treated 24-h NTN. (**h**) Graph showing semi-quantitative scoring of glomerular fibrinogen staining. (**a–c** and **e–g**) Original magnification, × 400. [+]$P < 0.05$, [++]$P < 0.01$ and [+++]$P < 0.001$ *vs* normal; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* SYKi.

P-selectin

Fibrinogen

a

e

b

f

c

g

d

P-selectin

h

Fibrinogen
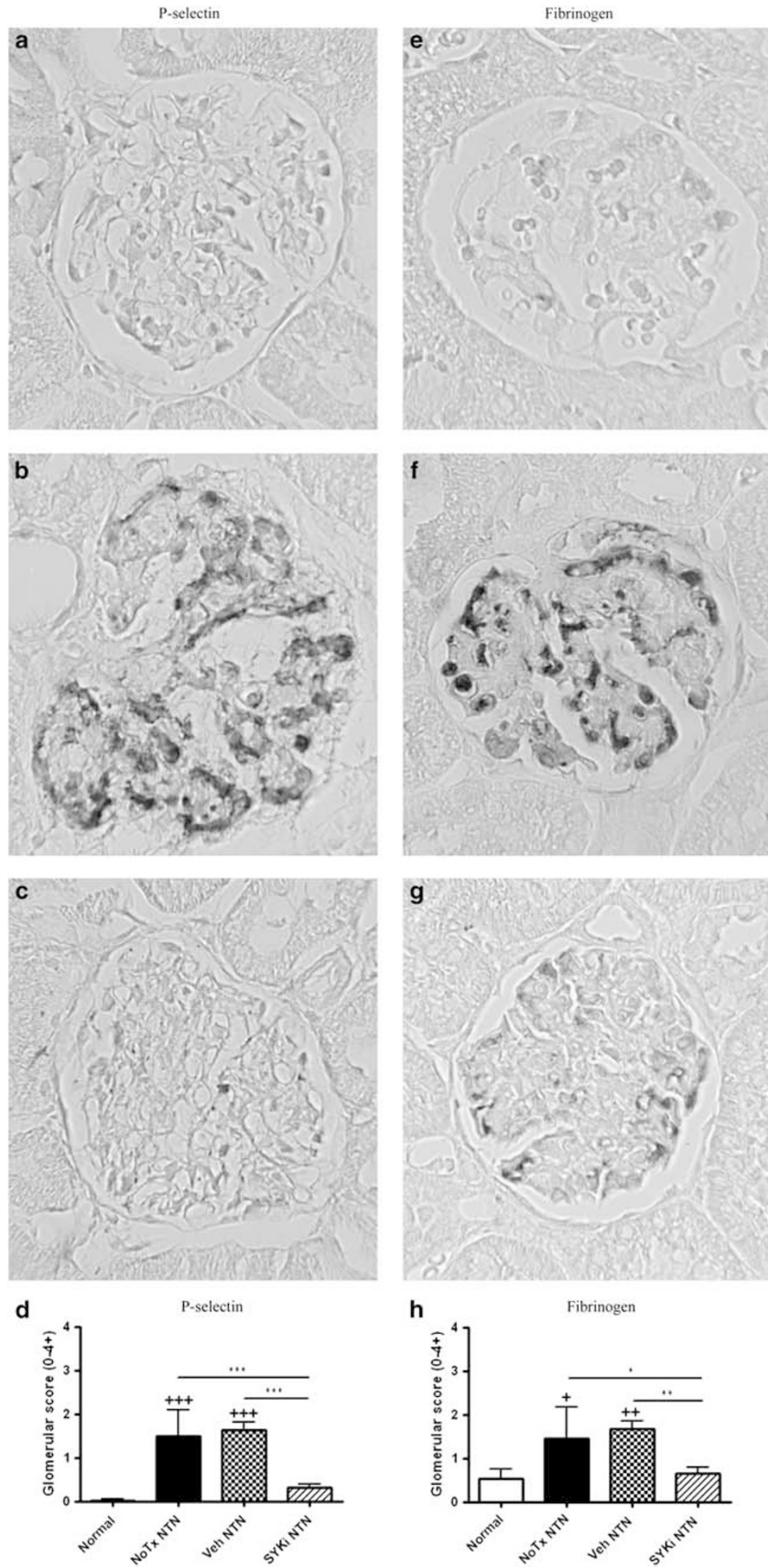
follows: nephrin (forward: 5′-CCATTACCATCCTGGGATC
T-3′; reverse: 5′-CCATCCATGACTGTCTCATCT-3′; probe:
5′-TGACCCTTTGCTGCCT-3′) and podocin (forward: 5′-CA
TCAAAGTGGAGAGAACTGAA-3′; reverse: 5′-ACAGAATC
TCAGCCGCCAT-3′; probe: 5′-CACAAAGACAGGCCAA-3′).
Primers and probes for NOS2, TNF-α, MMP-12 and CCL2
have been described previously.[22] The relative amount of
mRNA was calculated using comparative Ct (ΔΔCt) method.
All specific amplicons were normalised against 18S
RNA, which was amplified in the same reaction as an
internal control using commercial assay reagents (Applied
Biosystems).

Western Blot
Isolated glomeruli were lysed in 1 ml of RIPA buffer, cen-
trifuged and the supernatant was stored as described
previously.[23] Lysates were separated by SDS-PAGE using 12%
acrylamide gels, electroblotted onto a PVDF membrane, and
the blots were incubated for 1 h with Odyssey blocking buffer
(Millennium Science, VIC, Australia). Blots then were
incubated with the antibody of interest in blocking buffer
overnight at 4 °C. Blots were washed and then incubated with
an Alexa Fluor-conjugated secondary antibody for 1 h at
room temperature, washed and scanned using the Odyssey
infrared Imaging System (Licor Biosciences). Membranes
were re-probed with anti-α₁ tubulin to determine equiva-
lence of protein loading. Densitometry analysis was per-
formed using the Gel Pro analyser Program (Media
Cybernetics, Silver Spring, MD, USA).

Statistics
Data are presented as mean ± s.d. Analysis between groups of
animals was performed by parametric ANOVA using
Bonferroni's post-test for multiple comparisons. Non-para-
metric data were analysed by the Kruskal–Wallis ANOVA by
ranks using Dunn's post-test for multiple comparisons
(GraphPad Prism 5.0 Software, San Diego, CA, USA).

RESULTS
Syk is Activated in Rat NTN
Activation of Syk occurs by autophosphorylation of Tyr 525/
526 in the activation loop.[24] Using immunohistochemistry,
we identified p-Syk (Tyr 525/526) in glomerular cells at 3 h in
vehicle-treated rat NTN, whereas staining was absent in
normal glomeruli (Figures 1a and b). These p-Syk positive
cells are mostly within capillary loops and the staining
co-localised with infiltrating RP1+ neutrophils (Figures 1b
and c). Immunostaining for p-Syk was also seen within
capillary loops at 24 h in this model (Figure 1e). The
glomerular p-Syk staining at 3 h and 24 h of NTN was largely
abrogated by SYKi treatment (Figures 1d and f).

SYKi Prevents Proteinuria
All untreated and vehicle-treated animals developed protei-
nuria by 24 h of NTN (Figure 2a). The induction of

proteinuria was associated with a reduction in the number
of WT-1+ podocytes and downregulation of mRNA levels of
the podocyte-specific molecules, nephrin and podocin
(Figures 2b–d). SYKi treatment prevented the development
of proteinuria (Figure 2a). SYKi treatment provided partial
protection against podocyte loss, and SYKi prevented
the reduction in mRNA levels of nephrin and podocin
(Figures 2b–d).

SYKi Prevents Glomerular Thrombosis and Platelet Activation
Glomerular injury was evident in vehicle and untreated NTN
at 24 h as shown by glomerular hypercellularity and focal and
segmental capillary thrombosis (Figures 3a and b). There was
relatively little tubulointerstitial damage apart from small
numbers of protein casts. Platelet and endothelial activation
in NTN was evident by the strong immunostaining for
P-selectin and fibrinogen within glomerular capillary loops
(Figure 4).

SYKi treatment gave profound protection from glomerular
damage in rat NTN, with abrogation of capillary thrombosis
and only mild glomerular hypercellularity evident (Figures 3c
and d). SYKi treatment reduced immunostaining for
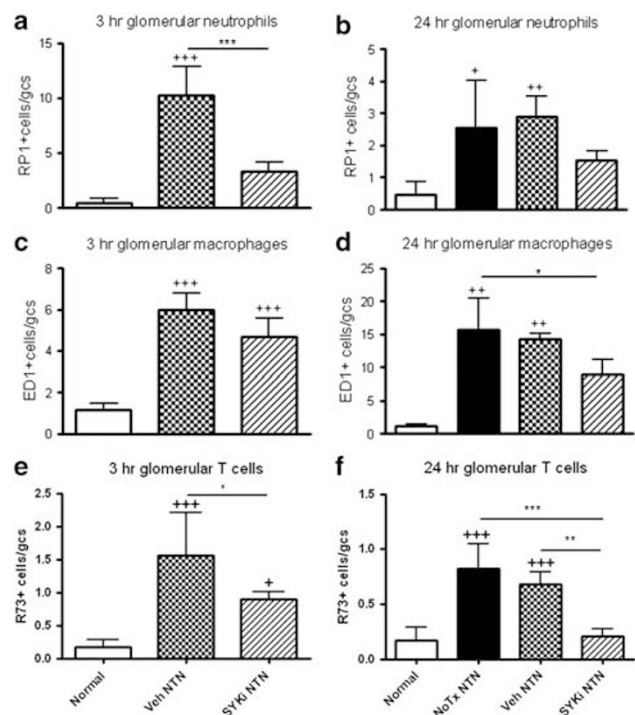P-selectin and fibrinogen by 81% and 61%, respectively

Figure 5 Quantification of glomerular leukocytes in NTN. (a–c) Graphs showing cell counts per glomerular cross-section (gcs) in normal rats and at 3 h NTN with vehicle or SYKi treatment for (a) RP1+ neutrophils, (b) ED1+ macrophages and (c) R73+ T cells. (d–f) Graphs showing cell counts in normal rats and at 24 h NTN with vehicle or SYKi treatment or no treatment (no Tx) for (d) RP1+ neutrophils, (c) ED1+ macrophages and (f) R73+ T cells. $^+P<0.05$, $^{++}P<0.01$ and $^{+++}P<0.001$ vs normal; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs SYKi.

(Figures 4). Taken together, these data suggest that SYKi treatment significantly reduced both platelet and endothelial activation. Glomerular deposition of sheep IgG, rat IgG and rat C3 at 24 h was unaltered by SYKi compared with vehicle and untreated NTN (data not shown).

SYKi Reduced Glomerular Leukocyte Recruitment

Consistent with previous studies in this model of rat NTN,[4,5,25] glomerular RP1+ neutrophil infiltration peaks ~3 h after anti-GBM serum administration in untreated and vehicle animals and is largely resolved by 24 h. Glomerular macrophage infiltrate is seen at 3 h and increases further at 24 h, while the T cell infiltrate peaks at 3 h and is partially reduced by 24 h (Figure 5). SYKi treatment substantially reduced the peak of glomerular neutrophil influx at 3 h compared with vehicle-treated animals (Figure 5a). Glomerular T cell infiltration was reduced by SYKi treatment at 3 and 24 h, while there was only a borderline effect upon the macrophage infiltrate (Figure 5). In addition, SYKi treatment did not affect white blood cell counts, which showed a trend towards neutrophilia in all groups with acute NTN (data not shown).

SYKi Inhibited Activation of JNK and p38 MAPK

We examined whether SYKi treatment modulates activation of the JNK and p38 MAPK pathways in rat NTN. Western blotting showed a significant increase in phosphorylation of JNK and p38 in isolated glomeruli in vehicle and untreated NTN, and this activation was prevented by SYKi treatment (Figure 6). Immunostaining identified a marked increase in the number of p-JNK+ cells and p-p38+ cells in glomeruli in vehicle and untreated NTN, and this response was

essentially abrogated by SYKi treatment (Figure 7). These results demonstrate that SYKi treatment prevents activation of JNK and p38 MAPK pro-inflammatory pathways in rat NTN.

SYKi Inhibits Glomerular Inflammation

The inflammatory response in isolated glomeruli was examined by real-time RT-PCR. Vehicle and untreated animals showed a robust upregulation of TNF-α, NOS2, MMP-12 and CCL2 mRNA levels at 3 and 24 h of NTN. SYKi substantially reduced mRNA levels of all of these pro-inflammatory molecules at 3 and 24 h (Figure 8).

Syk is Activated in Human Post-Infectious Glomerulonephritis

We sought to determine the relevance of these experimental findings to human acute neutrophil-mediated glomerular injury. We examined two cases of post-infectious glomerulonephritis, a disease generally associated with glomerular IgG and IgM deposition and a neutrophil-rich leukocytic infiltrate. No p-Syk staining was seen in cases of minimal change and thin membrane disease, which were chosen to represent 'normal' kidney without antibody deposition or leukocyte infiltration (Figure 9a). In both cases of post-infectious glomerulonephritis, numerous p-Syk+ cells were evident in glomeruli as illustrated in Figure 9b. Immunostaining of serial sections demonstrated that these glomerular p-Syk+ cells co-localise with infiltrating neutrophils (Figures 9c and d), showing a focal glomerular staining pattern similar to that seen in rat NTN (Figure 1). Furthermore, immunostaining for p-JNK and p-p38 followed a similar pattern to that of p-Syk in post-infectious glomerulonephritis (Figures 9e and f).
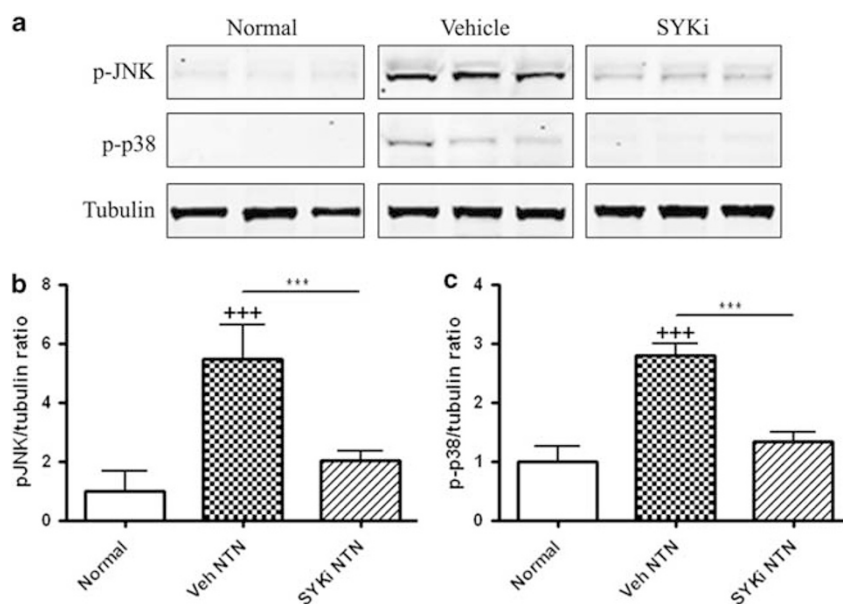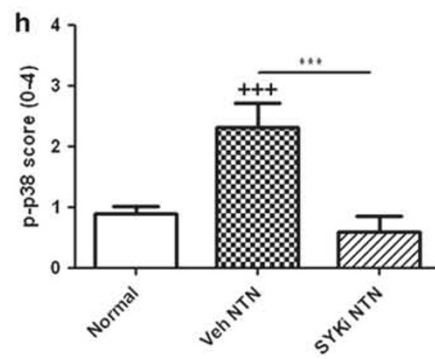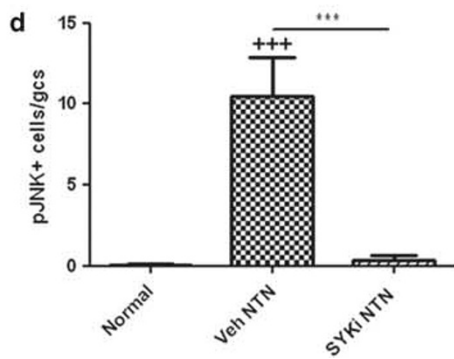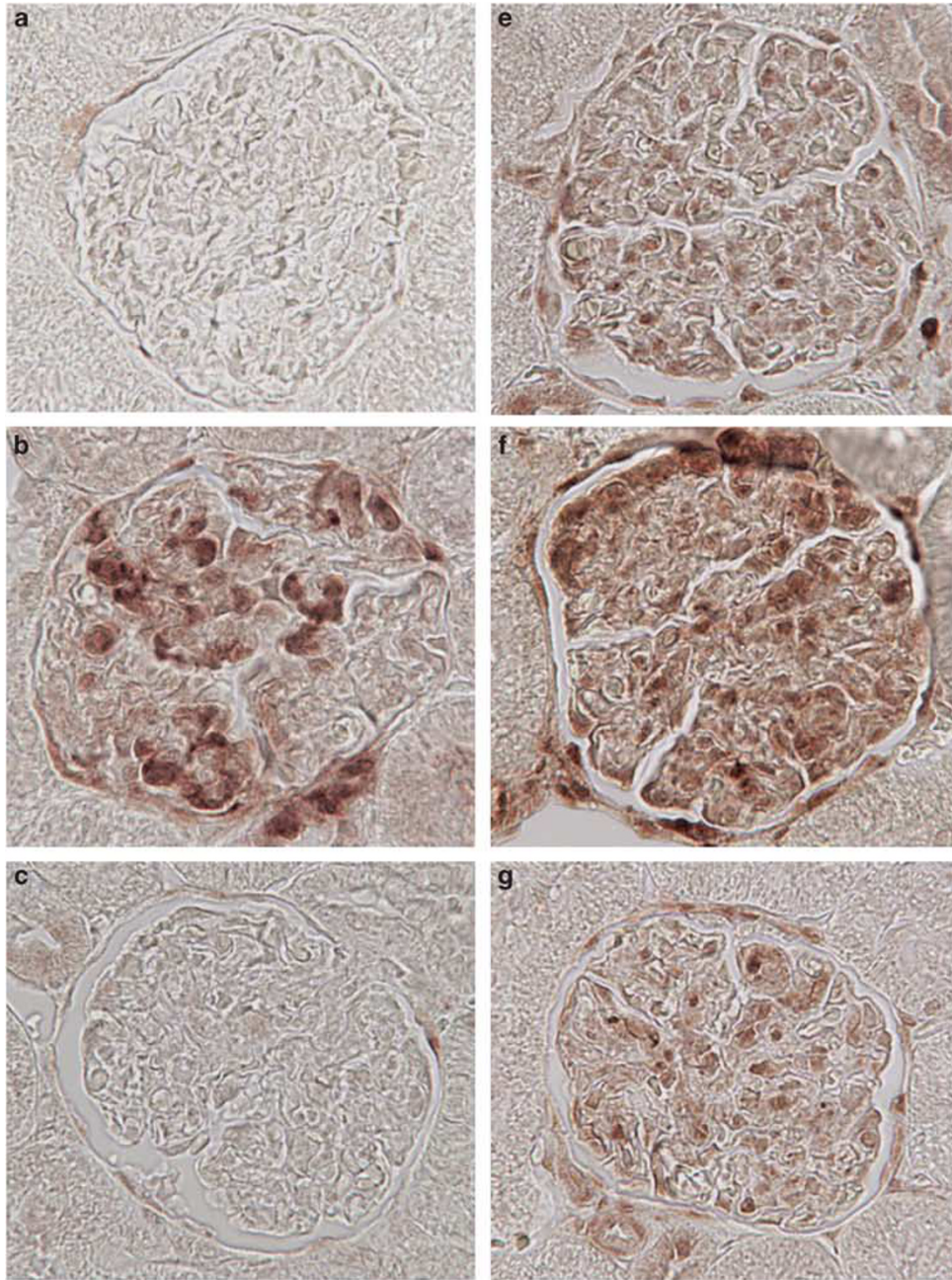

Figure 6 JNK and p38 activation in NTN. (**a**) Isolated glomeruli from normal rats, vehicle treated and SYKi treated 24-h NTN were analysed by western blotting for p-JNK and p-p38 and blots reprobed for tubulin. Graphs showing quantification of (**b**) p-JNK and (**c**) p-p38. $^{+++}P < 0.001$ *vs* normal; ***$P < 0.001$ *vs* SYKi.

DISCUSSION

This study has three novel findings: detection of Syk activation in human and experimental kidney disease, demonstration that a Syk inhibitor drug can prevent acute neutrophil-mediated glomerular injury and identification of Syk as an important upstream activator of JNK and p38 signalling pathways in acute glomerulonephritis.

It is well established that Syk has an important role in Fc-R-mediated activation of neutrophils, monocytes and mast cells;[11] however, direct observation of Syk activation *in vivo* has been lacking. In this study, we have used

Figure 8 Real-time RT-PCR of isolated glomeruli from normal and NTN rats. (**a–d**) Analysis of relative mRNA levels in normal rats and in vehicle and SYKi-treated animals at 3 h NTN for (**a**) TNF-α, (**b**) NOS2, (**c**) MMP-12 and (**d**) CCL2. (**e–h**) Analysis of relative mRNA levels in normal rats and in vehicle-treated, SYKi-treated and untreated animals at 24 h NTN for (**e**) TNF-α, (**f**) NOS2, (**g**) MMP-12 and (**h**) CCL2. $^{++}P < 0.01$ and $^{+++}P < 0.001$ *vs* normal; $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs* SYKi.

immunohistochemistry to identify Syk activation on the basis of phosphorylation of its activation loop. Glomeruli in normal kidney showed no evidence of Syk activation, but p-Syk staining was evident within glomerular capillaries in acute NTN in a pattern suggesting activation in neutrophils, platelets and macrophages. Importantly, we also observed Syk activation in biopsies of post-infectious glomerulonephritis in a staining pattern, which co-localised with infiltrating neutrophils, whereas minimal change disease and thin membrane disease showed no evidence of Syk activation. These findings open the way for a detailed examination of Syk activation in human glomerular diseases.

It is well established that glomerular antibody deposition can induce acute renal injury via Fcγ-receptor-dependent activation of platelets and neutrophils.[2,3,19,26] Syk inhibitors have been shown to suppress macrophage-mediated renal injury,[17,18,27] but whether Syk is also involved in neutrophil-dependent glomerular injury is unknown. In this study, blockade of Syk signalling using SYKi reduced podocyte damage and prevented proteinuria. There are several potential mechanisms, which may, individually or in combination, have contributed to this protective effect.

Firstly, SYKi treatment substantially reduced the peak of glomerular neutrophil influx at 3 h, which is critical for induction of acute proteinuria in this disease model.[19] This inhibition of neutrophil influx could be due to several mechanisms, although depletion of circulating neutrophils and platelets was ruled out. Platelets have an important role in glomerular neutrophil recruitment and the development of acute proteinuria,[26,28] and blockade of P-selectin (CD62P) has been shown to prevent glomerular neutrophil recruitment and acute proteinuria.[28,29] In our study, untreated and vehicle-treated NTN showed extensive glomerular capillary thrombi, together with P-selectin expression and fibrin deposition. SYKi treatment provided marked protection from histological damage, as well as abrogating glomerular P-selectin and fibrinogen staining. Thus, blockade of platelet activation and/or P-selectin expression are likely causes of reduced neutrophil influx. Indeed, a role for Syk has been described in signal transduction by the major P-selectin ligand expressed by leukocytes, P-selectin glycoprotein ligand 1.[30,31] Syk signalling also has an important role in platelet activation via the Fc-Rγ associated receptor, GP-VI.[32] In addition, Syk signalling has been implicated in integrin signalling, both in platelet activation and in neutrophil adhesion.[33,34]

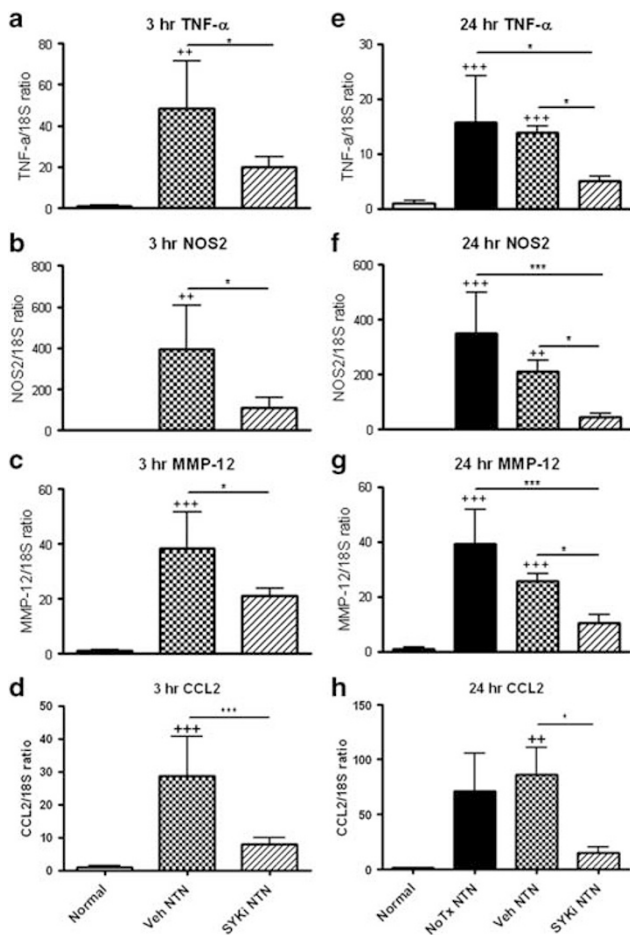Secondly, SYKi treatment suppressed glomerular inflammation at the 3 h time point as shown by the reduction

Figure 7 Immunostaining for JNK and p38 activation in NTN. (**a**) p-JNK immunostaining is absent in normal glomeruli. (**b**) Glomerular p-JNK staining is seen in capillary loops in 3-h vehicle rat NTN. (**c**) SYKi treatment abolished glomerular p-JNK in NTN. (**d**) Graph showing quantification of the number of p-JNK+ cells per glomerular cross-section (gcs). (**e**) Some p-p38+ cells are seen in normal rat glomeruli. (**f**) Vehicle-treated rat (3 h) NTN showing an increase in p-p38+ cells. (**g**) SYKi treatment of NTN reduces glomerular p-p38+ cells. (**h**) Graph showing semi-quantitative scoring of glomerular p-p38+ cells. $^{+++}P < 0.001$ *vs* normal; $^{***}P < 0.001$ *vs* SYKi.

in mRNA levels for individual pro-inflammatory mediators such as TNF-α, blockade of which has been shown to suppress acute proteinuria in the first 24 h in this disease model.[35] Of note, SYKi treatment also markedly suppressed expression of pro-inflammatory molecules at the 24 h time point in rat NTN when the neutrophil influx has largely
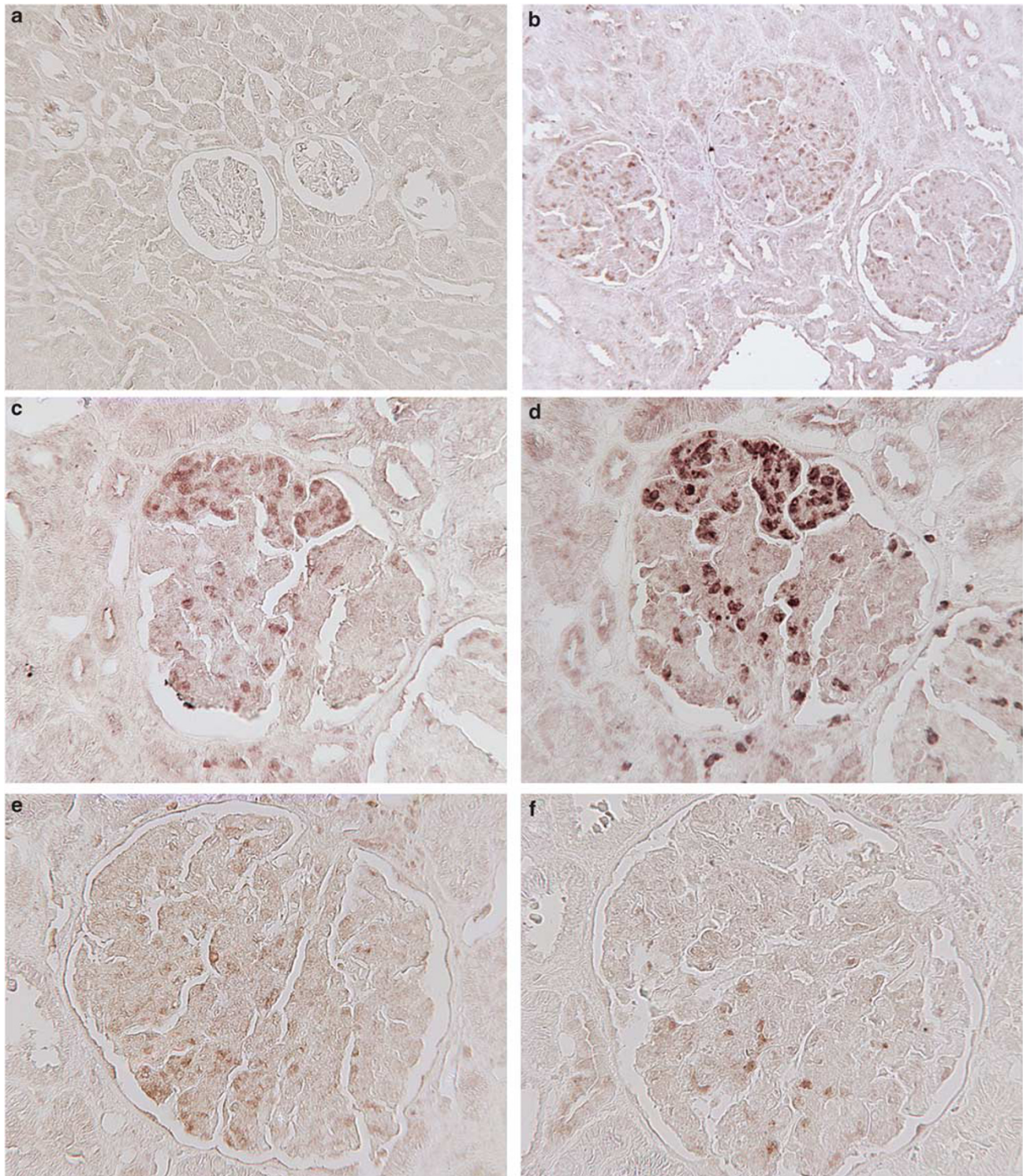

Figure 9 Syk activation in human kidney disease. (**a**) Minimal change disease has no immunostaining for p-Syk (Tyr 525/526). (**b**) A case of post-infectious glomerulonephritis shows p-Syk + cells in glomeruli. Serial sections in post-infectious glomerulonephritis shows a focal glomerular pattern of p-Syk + cells (**c**), which co-localises with neutrophils within capillary loops (**d**). In the same case, immunostaining shows the presence of (**e**) p-JNK + cells, and (**f**) p-p38 + cells, which have a similar glomerular distribution as that of p-Syk + cells. Original magnification, × 160 (**a**, **b**), × 400 (**c–f**).

resolved and monocytes/macrophages are the main glomerular leukocyte population. Thus, it can be inferred that although SYKi treatment had only a minor effect upon macrophage accumulation at 24 h, it had a profound inhibitory effect upon activation of the macrophage infiltrate, with suppression of macrophage-specific effector molecules such as NOS2 and MMP-12.[22,36,37] This is consistent with a previous study showing that a Syk inhibitor drug suppressed the macrophage-dependent phase of renal injury in NTN.[18]

Thirdly, an important mechanism through which SYKi treatment suppressed acute renal injury in rat NTN is the inhibition of p38 and JNK signalling. *In vitro* studies have linked Fc-Rγ activation to p38 and JNK signalling in mast cells,[38] but there is little data to demonstrate such a Syk-p38/JNK signalling pathway *in vivo*. In this study, we identified activation of Syk, p38 and JNK in infiltrating glomerular neutrophils in rat NTN, and demonstrated that SYKi treatment resulted in suppression of p38 and JNK activation. Furthermore, investigation of post-infectious glomerulonephritis identified glomerular activation of Syk, p38 and JNK, which exhibited similar immunostaining patterns, consistent with being activated in infiltrating neutrophils, although further work needs to be done to define Syk activation is a much wider range of human kidney diseases.

We have previously demonstrated that blockade of p38α signalling significantly inhibits acute proteinuria in the first 24 h in this disease model.[5] This protective effect was attributed to inhibition of glomerular platelet activation and neutrophil influx at the 3 h time point. Indeed, the inhibition of P-selectin immunostaining seen with SYKi treatment in the current study parallels that observed previously with p38α blockade.[5] JNK signalling also has a role in the induction of acute proteinuria in the first 24 h of rat NTN. Administration of a selective JNK inhibitor drug largely abrogated proteinuria in the first 24 h of rat NTN, although this did not affect the neutrophil influx at 3 h.[4] These studies, including direct addition of a JNK inhibitor to macrophages before adoptive transfer, have established that JNK signalling in macrophages is not essential for recruitment to the inflamed glomerulus, but that JNK signalling is required for activation of the macrophage pro-inflammatory response.[4,22,39,40] The finding in the current study that SYKi treatment caused only a minor reduction in glomerular macrophages at 24 h in NTN, but yet markedly suppressed macrophage activation is consistent with the known effects of inhibition of JNK activation. Thus, the current study provides several lines of evidence to argue that Syk signalling is required for activation of the p38 and JNK pathways, and that this represents an important pathway of acute neutrophil-mediated glomerular injury.

One limitation in this type of study is the question of specificity of kinase inhibitor molecules. SYKi does have some activity against Flt-3, KDR and JAK-2 kinases, which could contribute to the efficacy of SYKi treatment, although SYKi has no activity against p38 or JNK. Ultimately, only a

genetic approach using conditional deletion of the *Syk* gene can formally prove a pathogenic role of Syk in glomerulonephritis.

In summary, we have identified neutrophil Syk activation in acute experimental antibody-dependent glomerular injury and confirmed the relevance of this finding in human acute post-infectious glomerulonephritis. This study provides several lines of evidence to argue that Syk signalling is required for activation of the p38 and JNK pathways, and that this represents an important pathway of acute neutrophil-mediated glomerular injury in antibody-dependent kidney disease. These results identify Syk as a potential therapeutic target in antibody-dependent kidney disease such as the platelet and neutrophil-dependent glomerular injury in ANCA vasculitis.

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