

An inhibitor of spleen tyrosine kinase suppresses experimental crescentic glomerulonephritis

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Abstract

Non-selective inhibitors of spleen tyrosine kinase (SYK) efficiently suppress disease in T cell-dependent models of crescentic glomerulonephritis. However, the therapeutic potential of selective SYK inhibitors in this disease has not been established. In addition, we lack knowledge regarding SYK expression in non-myeloid cells in glomerulonephritis. We addressed these two issues in a rat model of nephrotoxic serum nephritis (NTN) using a SYK inhibitor, GS-492429. Disease was induced in Sprague-Dawley rats (Study 1) or Wistar-Kyoto (WKY) rats (Study 2) by immunization with sheep IgG and administration of sheep anti-rat nephrotoxic serum. Animals were untreated or received GS-492429 (30 mg/kg/bid) or vehicle treatment from 2 h before nephrotoxic serum injection until being killed 3 or 24 h later (Study 1) or 14 days later (Study 2). Two-colour confocal microscopy found that SYK expression in NTN kidney was restricted to myeloid cells and platelets, with no evidence of SYK expression by T cells, mesangial cells, podocytes or tubular epithelial cells. In Study 1, GS-492429 treatment significantly reduced glomerular neutrophil and macrophage infiltration, with protection from glomerular thrombosis and proteinuria. In Study 2, GS-492429 treatment reduced glomerular crescent formation by 70% on day 14 NTN in conjunction with reduced glomerular thrombosis, glomerulosclerosis and tubular damage. This was accompanied by a marked reduction in markers of inflammation (CCL2, TNF- α , NOS2, MMP-12). Importantly, the protective effects of GS-492429 were independent of T cell infiltration and activation and independent of JAK/STAT3 signalling. In conclusion, this study demonstrates that a SYK inhibitor can suppress the development of crescentic glomerulonephritis through effects upon myeloid cells and platelets.

Keywords

crescent, glomerulonephritis, macrophage, STAT3, SYK, T cell

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Introduction

Rapidly progressive glomerulonephritis (RPGN) is a major medical issue in which patients can lose renal function and progress to require dialysis within weeks to months.¹ The formation of glomerular crescents is a hallmark of this diverse group of antibody-dependent diseases which includes anti-glomerular basement membrane (GBM) disease, Class IV lupus nephritis, anti-neutrophil nuclear antigen-induced

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vasculitis and crescentic IgA nephropathy.¹ Current treatment is largely limited to general immunosuppressive agents such as steroids, cyclophosphamide and mycophenolate; thus, there is a pressing need for more selective therapies.^{2,3}

Multiple immune cell types are involved in the pathogenesis of this group of antibody-dependent diseases, including T and B cells, macrophages and neutrophils.^{1,4} Experimental studies have shown the importance of myeloid cell activation via Fcγ receptors in causing renal injury in models of anti-GBM disease,^{5–7} while genetic studies have shown that copy number polymorphism in Fcγr3 predisposes humans and rats to glomerulonephritis.⁸

Spleen tyrosine kinase (SYK) is an intracellular kinase that is expressed by most leukocyte populations except mature T cells.⁹ SYK plays important roles in signalling via the B cell receptor, Fc receptors and the dectin-1 cell surface receptor.⁹ In addition, SYK plays an important role in platelet activation via platelet glycoprotein VI (GPVI) and the common FcR chain.¹⁰ Activation of SYK in myeloid cells has been identified in human biopsy studies of crescentic glomerulonephritis, while conditional *Syk* gene deletion in myeloid cells is protective in a mouse model of anti-GBM disease,^{11,12} establishing SYK as a therapeutic target in RPGN.

Many inhibitors of the kinase activity of SYK have been developed with the most widely studied compound being R788 (also known as fostamatinib).¹³ R788 is remarkably effective in suppressing animal models of lupus nephritis and anti-GBM disease.^{14–17} However, this drug inhibits many kinases apart from SYK.¹⁸ In particular, R406 (the active metabolite of R788) inhibits JAK2 > JAK1 > SYK > JAK3.^{13,19,20} This may explain the ability of R788 to inhibit T cell activation in vitro and in vivo given that T cell activation via interleukin (IL)-2 operates mostly through JAK1 and JAK3, while IL-12-induced T cell activation operates through JAK2.²¹ T cells play an important role in the development of crescentic kidney disease in models of lupus nephritis and anti-GBM disease.^{22–25} Thus, it is unclear whether the protective effects of R788 in these models relate primarily to inhibition of T cell activation or to blockade of SYK signalling.

A second question regarding the role of SYK in RPGN relates to precisely which cell types express SYK in the injured kidney? SYK has been reported

to be expressed by a variety of non-leukocytes including smooth muscle cells, fibroblasts, epithelial cells, mesangial cells and podocytes.^{26–30} SYK expression is evident in myeloid cells and platelets in human kidney disease;^{11,12} however, SYK expression in other cell types in the injured kidney is not well characterized.

In this study, we sought to (1) investigate whether the use of a pharmacologic SYK inhibitor could significantly reduce the development of experiment crescentic glomerulonephritis without affecting the T cell response or JAK/STAT signalling and (2) investigate the cellular expression of SYK in non-myeloid cells. To achieve this, we used a SYK inhibitor, GS-492429, which has more than 20-fold selectivity for SYK over all other kinases, in rat models of nephrotoxic serum nephritis (NTN).

Materials and methods

Antibodies and reagents

Mouse monoclonal antibodies were used as follows: CD11b/c (OX-42), CD68 (ED1), T cell receptor (R73), CD90 (OX-7/Thy-1), endothelium (RECA-1; all Dako, Glostrup, Denmark), granulocytes (RP-1; BD Pharmingen, North Ryde, NSW, Australia), anti-tubulin (Abcam, Cambridge, UK), and rabbit monoclonal antibodies to SYK (D3ZIE) and phospho-STAT3 (Tyr705; Cell Signalling, Boston, MA, USA). Polyclonal antibodies used were goat anti-collagen IV (Southern Biotechnology, Birmingham, AL, USA), rabbit anti-Wilms tumour 1 (WT-1) antigen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-fibrinogen (Santa Cruz Biotechnology), rabbit anti-phospho-SYK (Tyr525,526, Cell Signalling), goat anti-synaptopodin (Santa Cruz Biotechnology) and fluorescein isothiocyanate (FITC)-conjugated rabbit antibodies to sheep IgG, rat IgG and rat C3 (Dako). Secondary antibodies used were Alexa Fluor 568 Donkey anti-mouse IgG, Alexa Fluor 594 Donkey anti-rabbit IgG, Alexa Fluor 488 Donkey anti-rabbit IgG, Alexa Fluor 680 goat anti-rabbit IgG and IRDye 800 donkey anti-mouse IgG (Thermo Fisher Scientific, Eugene, OR, USA). GS-492429 is an adenosine triphosphate (ATP)-competitive inhibitor of SYK inhibitor provided by Gilead Sciences. GS-492429 has been described (compound 55)¹⁹ and inhibits SYK with a K_d of 9.5 nM and has more than 20-fold selectivity for SYK compared to a panel of 400 kinases (see Supplementary Table 1).

Rat NTN (studies 1 and 2)

Study 1. NTN was induced in inbred female Sprague-Dawley rats (150–200 g; Monash Animal Services, Melbourne). Groups of eight rats were immunized with 1 mg of sheep IgG in Freund's complete adjuvant followed 5 days later (day 0) by tail vein injection of sheep anti-rat GBM serum and killed 3 or 24 h later as previously described.³¹ Animals were given GS-492429 (30 mg/kg twice a day) or vehicle alone (Cremophor EL/ethanol/sodium chloride) by oral gavage at 2 h before anti-GBM serum injection. Groups killed at 3 h had only a single treatment, while groups killed at 24 h had two additional gavages at 10 and 22 h.

Study 2. NTN was induced in male WKY rats (180–200 g; Animal Resource Centre, Perth, Australia) by immunization with 1 mg of sheep IgG in Freund's complete adjuvant followed 5 days later (day 0) by tail vein injection of sheep anti-rat GBM serum as previously described.³² Animals were given GS-492429 (30 mg/kg twice a day), vehicle alone or no treatment (No Tx), by oral gavage beginning 2 h before anti-GBM serum injection and continued until rats were killed on day 14, with the final treatment given 2 h before killing. All animal experiments were approved by Monash Medical Centre Animal Ethics Committee.

Assessment of proteinuria and serum creatinine

Urinary protein excretion was measured using a Coomassie protein assay kit (Thermo Fisher Scientific). Serum creatinine was measured using a Duppon ARL analyser by Department of Biochemistry, Monash Health.

Histology

Periodic acid-Schiff (PAS) staining was performed on 2 μ m sections of formalin-fixed tissue. Glomerular thrombosis was scored in the day 1 NTN model based on the area involved: 0, none; 1+, 1%–25%; 2, 25%–49%; and 3+, over 50%. The percentage of glomeruli with crescent formation was scored in the day 14 NTN model. Both analyses scored all full-sized glomerular cross-sections in each animal on blinded slides.

Immunohistochemistry

Immunoperoxidase staining for R73+ T cells, RP-1+ neutrophils and RECA-1+ endothelium was

performed on cryostat sections. Immunostaining for ED1, WT-1, collagen IV, α -smooth muscle actin (SMA), SYK and fibrinogen was performed on formalin-fixed paraffin sections as previously described.³² The number of glomerular R73+, RP-1+, ED1+ and WT-1+ cells was scored in all full-sized glomerular cross-sections in each animal on blinded slides. RECA-1 staining of capillaries in the glomerular tuft was scored as follows: 0, normal density of capillaries; 1+, reduced capillary density in <25% of tuft; 2+, reduced capillary density in 25%–50% of the tuft; and 3+, reduced capillary density in >50% of the tuft. The number of interstitial R73+ T cells was scored in high-power fields (\times 250) covering 90% of the cortex.

Confocal microscopy

Dual fluorescence labelling was performed on 6 μ m cryostat sections. After air-drying and blocking in 1% bovine serum albumin (BSA), sections were incubated with primary antibodies (rabbit anti-SYK together with other mouse or goat antibodies) followed by fluorescent-conjugated secondary antibodies. Finally, sections were incubated with 10 μ g/mL DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) for 5 min. Slides were mounted with anti-fade mounting medium (polyvinyl alcohol mounting medium with DABCO, Fluka) and then sealed with nail polish. Fluorescence images were taken on a Nikon C1 inverted microscope (Monash Micro Imaging Facility). The sections were scanned in one direction with excitation lasers of 488 and 561 nm. The fluorescence signals were collected under oil immersion with a \times 60 lens.

Immunofluorescence staining was performed on sections of snap-frozen tissue using FITC-conjugated antibodies to rat C3 and rat IgG and rat C3.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) was performed as described previously.³² Rat kidney cross-section samples were snap-frozen in liquid nitrogen and stored at -80°C until RNA was extracted using TRIzol[®]. Complementary DNA (cDNA) was prepared from total RNA by reverse transcription using random hexamer primers (Invitrogen, Carlsbad, California, USA). RT-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 (5-Fluorescein) -labelled minor groove binder probes were obtained from Applied Biosystems or designed as previously described.^{32,33} The relative amount of messenger RNA (mRNA) was calculated using the comparative Ct method. All specific amplicons were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, which was amplified in the same reaction as an internal control using commercial assay reagents (Applied Biosystems).

Statistics

Data are expressed as means ± standard deviation (SD). Data for groups of three or more were analysed by one-way analysis of variance (ANOVA) with post hoc analysis with Tukey's multiple comparison test or the Kruskal–Wallis test, except for day 14 proteinuria data which employed two-way ANOVA with Sidak's multiple test. Analysis was performed using GraphPad Prism 7.01 (GraphPad Software Inc, San Diego, CA, USA).

Results

Two models of NTN were investigated to analyse different mechanistic features of crescentic glomerulonephritis. Study 1 examined a 24-h NTN model in outbred Sprague-Dawley rats which features acute glomerular inflammation and kidney injury as shown by increased protein excretion in the urine.^{34,35} Study 2 examined a 14-day NTN model in WKY rats which features development of crescentic lesions; this model does not exhibit acute neutrophil-dependent proteinuria at 24 h, rather proteinuria develops around day 4.^{33,36}

SYK expression in rat NTN

Immunoperoxidase staining identified small numbers of SYK-expressing (SYK+) cells in the glomerulus and interstitium of normal rat kidney (Figure 1(a)). There was a clear increase in glomerular SYK staining on day 1 NTN which corresponded with the glomerular macrophage infiltrate and areas of thrombosis (Figure 1(c) and (d)). More extensive SYK staining was evident on day 14 NTN, both in glomeruli and crescents and in the interstitium, although tubules did not exhibit SYK

expression (Figure 1(e)). Much of the SYK staining on day 14 NTN corresponded to macrophages and to areas of thrombosis (Figure 1(e) and (f)). To examine this question in more detail, we performed two-colour confocal microscopy on day 14 NTN. Substantial co-localization of SYK and the macrophage marker, OX-42, was evident (Figure 2(a)). However, little or no co-localization of SYK was evident with R73+ T cells, OX-7+ mesangial cells or synaptopodin+ podocytes (Figure 2(b)–(d)).

GS-492429 prevented glomerular injury at 24 h of rat NTN

Vehicle-treated rats exhibited transient glomerular neutrophil infiltration at 3 h of NTN, a progressive increase in glomerular macrophages over 3 and 24 h, with glomerular thrombosis evident at 24 h, and significant proteinuria (Figure 3(a) and (c)–(f)). GS-492429 treatment (30 mg/kg/bid) starting 1 h before injection of anti-GBM serum into primed rats gave substantial protection from both glomerular thrombosis and proteinuria (Figure 3(b)–(d)). This protection was associated with reduced glomerular neutrophil infiltration at 3 h and reduced glomerular macrophage infiltration at 3 and 24 h (Figure 3(e) and (f)). No glomerular T cell infiltration was evident in either vehicle- or GS-492429-treated animals in 3 h or day 1 NTN (Figure 3(g)).

GS-492429 suppressed SYK activation and glomerular lesions on day 14 of rat NTN

Western blotting of whole kidney tissue identified a significant increase in phosphorylation (activation) of SYK in the untreated and vehicle-treated groups on day 14 NTN. This SYK activation was substantially suppressed by GS-492429 treatment (30 mg/kg/bid) starting 1 h before injection of anti-GBM serum into primed rats (Figure 4(a) and (b)).

The untreated and vehicle-treated groups developed severe glomerular damage, including thrombosis, atrophy and crescents on day 14 rat NTN (Figure 4(c)). Crescent formation was evident in 30%–40% of glomeruli in these groups (Figure 4(e)). The untreated and vehicle-treated groups also exhibited prominent glomerular fibrin deposition and a significant reduction in glomerular capillary density as demonstrated by the capillary rarefaction index (Figure 4(d), (f) and (g)). In addition, these groups exhibited glomerulosclerosis on

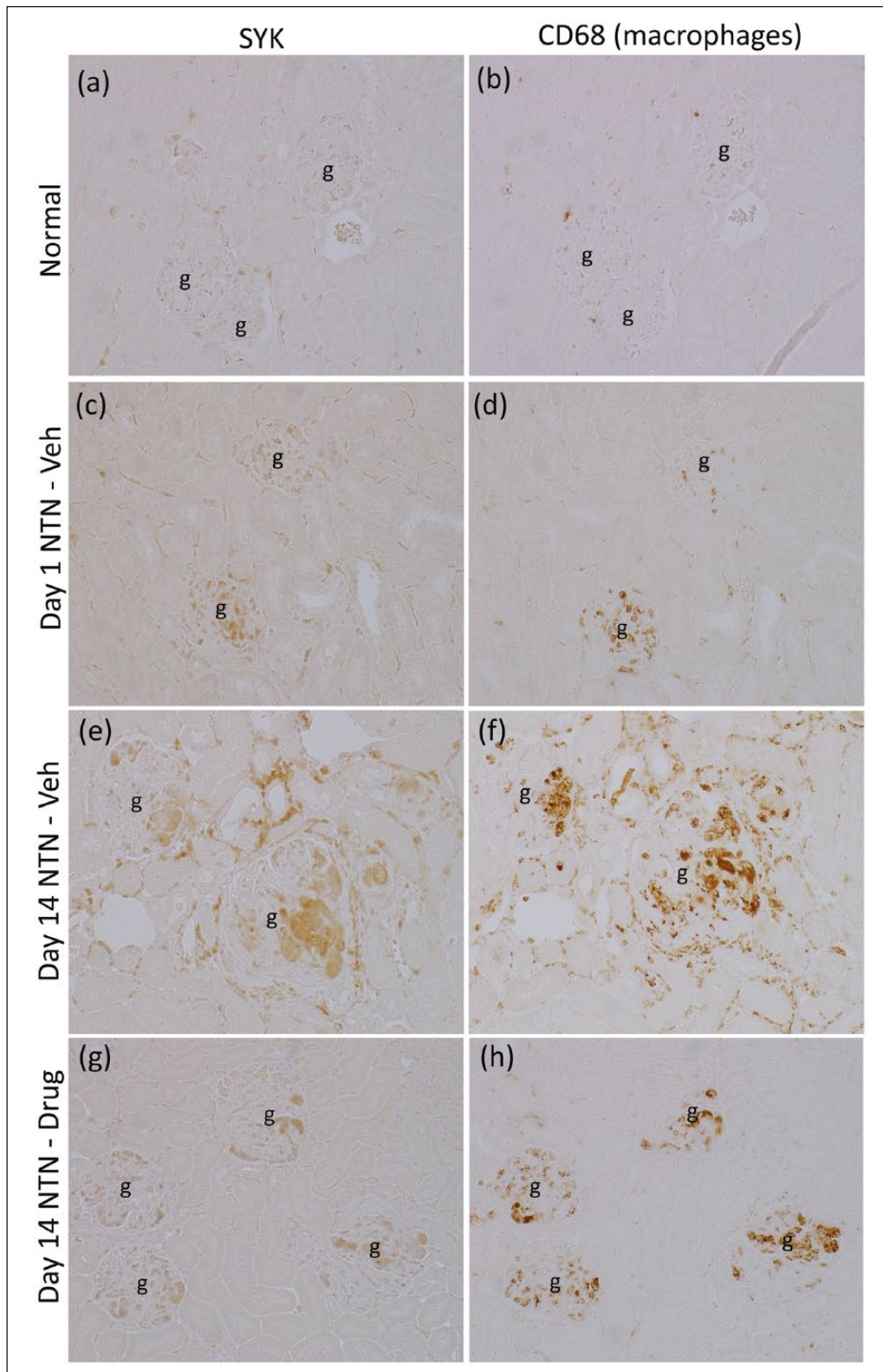


Figure 1. Immunostaining for SYK and CD68+ macrophages on serial sections. (a and b) Normal rat kidney shows small numbers of SYK+ cells and CD68+ macrophages, mostly in the interstitium. (c and d) Vehicle-treated day 1 NTN shows SYK staining in capillaries of the glomerulus (g) which is largely co-incident with glomerular infiltration of CD68+ macrophages. (e and f) Vehicle-treated day 14 NTN shows substantial glomerular SYK staining plus infiltration of SYK+ cells in the interstitium. The SYK staining is largely coincident with the CD68+ macrophage infiltrate and with diffuse staining in areas of thrombosis. (g and h) GS-492429-treated day 14 NTN shows reduced SYK staining that is largely restricted to glomeruli. The CD68+ macrophage infiltrate is also reduced in glomeruli and substantially reduced in the interstitium. Original magnification $\times 200$ (a–d, g and h); $\times 400$ (e and f).

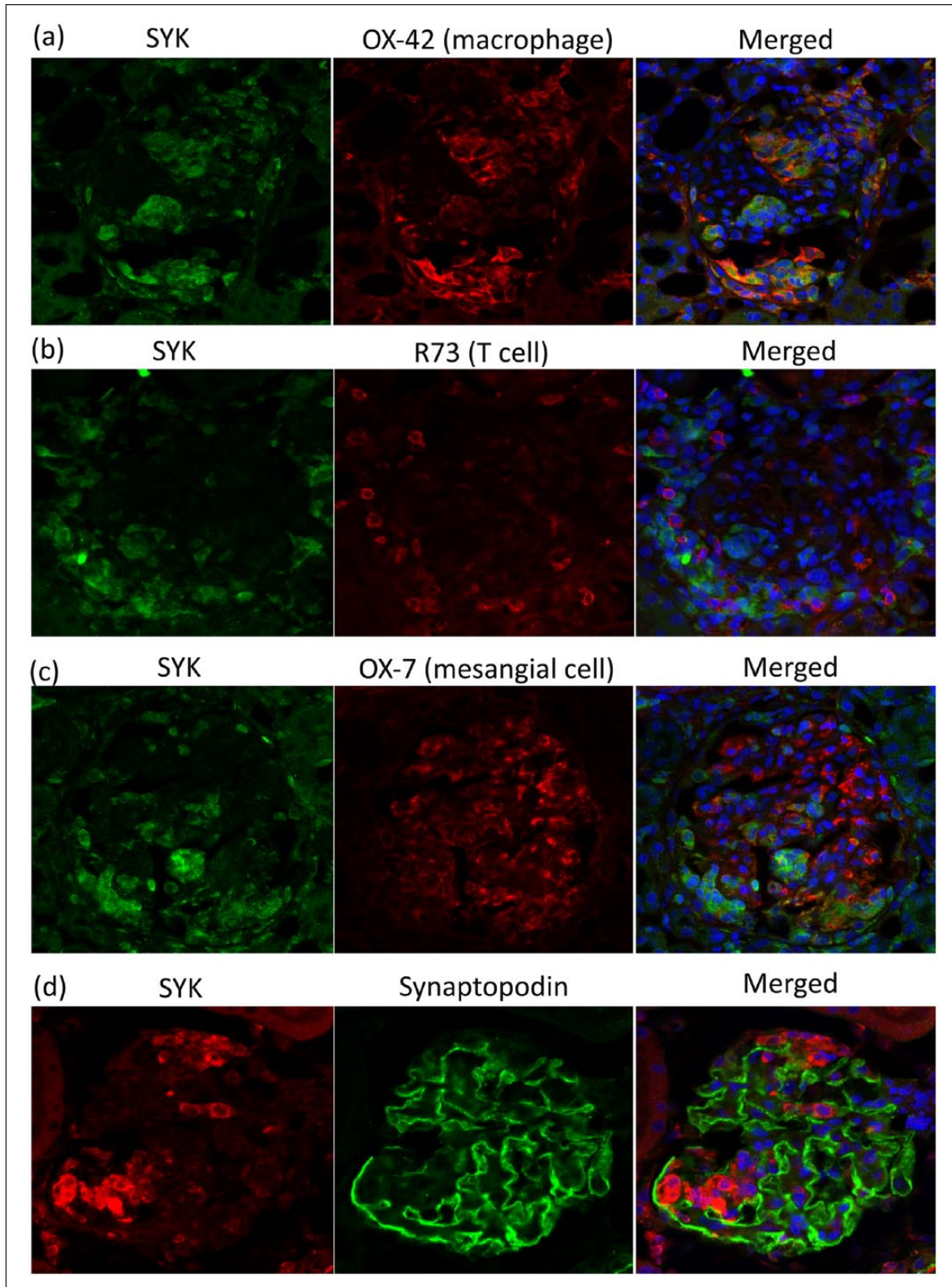


Figure 2. Analysis of SYK expression on day 14 of vehicle-treated NTN by confocal microscopy. (a) Double staining for SYK and OX-42+ macrophages shows considerable overlapping indicating that SYK expression on day 14 NTN is largely restricted to macrophages. (b) Double staining for SYK and R73+ T cells indicates no overlap. (c) Double staining for SYK and OX-7+ mesangial cells indicates no overlap. (d) Double staining for SYK and synaptopodin+ podocytes indicates no overlap. Original magnification $\times 600$.

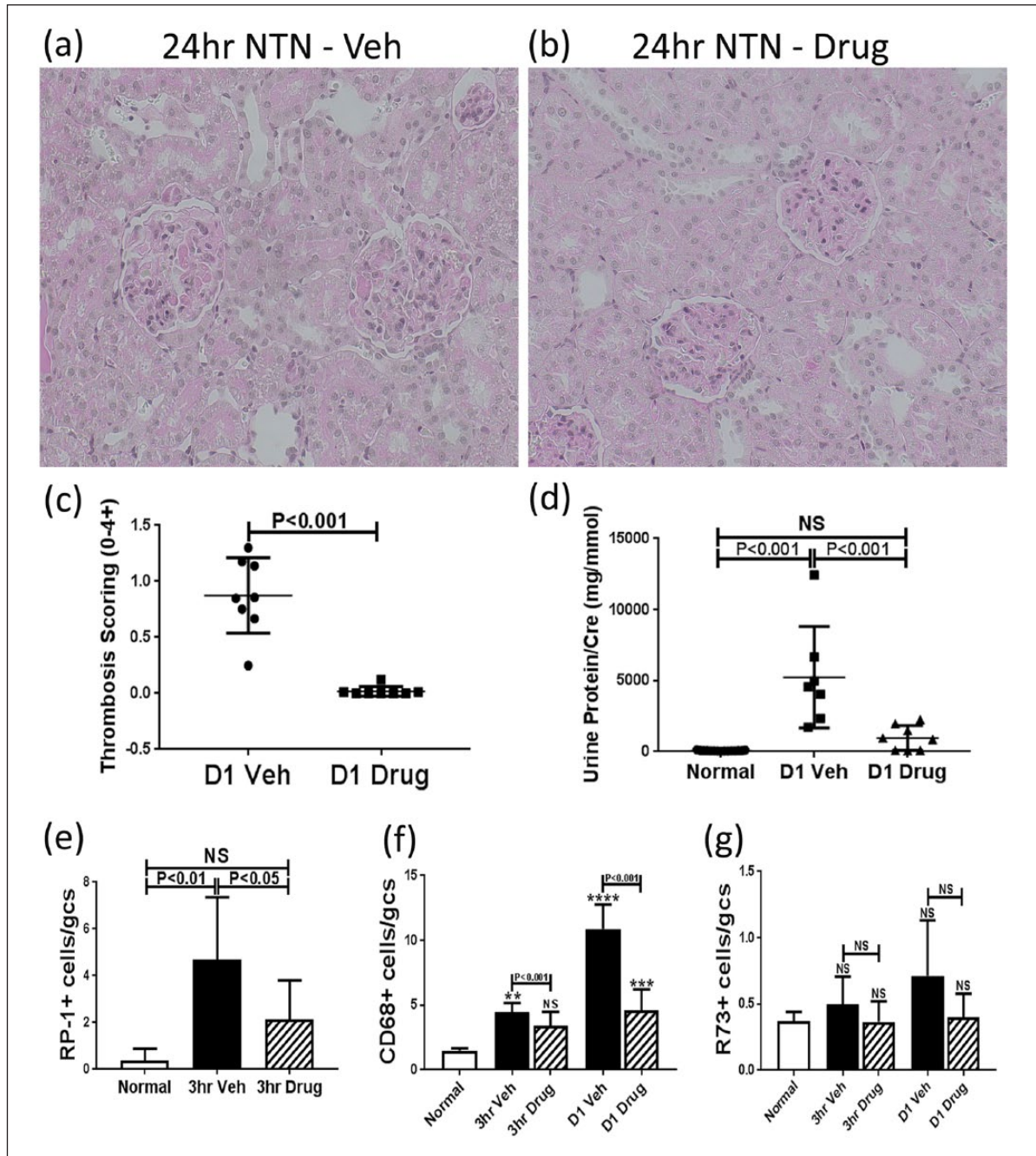


Figure 3. Effect of GS-492429 on leukocyte infiltration, proteinuria and thrombosis in acute (3 and 24 h) rat NTN. (a) PAS staining shows glomerular capillary thrombosis in day 1 of vehicle-treated NTN, which (b) is absent in GS-492429-treated NTN. (c) Quantification of glomerular capillary thrombosis on day 1 NTN. (d) Urinary protein-to-creatinine (Cr) ratio on day 1 NTN. Quantification of (e) glomerular RP-1+ neutrophils, (f) CD68+ macrophages and (g) R73+ T cells at 24 h and/or 3 h of NTN. Data are mean \pm SD. Original magnification \times 400 (a and b).

day 14 NTN based upon abnormal glomerular collagen IV deposition (Figure 5(a), (b), (d)) and increased mRNA levels of the pro-fibrotic molecules: collagen I, TGF- β and PAI-1 (Figure 5(d)–(g)). Furthermore, the untreated and vehicle-treated day 14 NTN groups exhibited tubulointerstitial damage in terms of mononuclear cell infiltration

and tubular damage (Figure 4(c)), and up-regulation of mRNA levels for the tubular damage marker, Kidney Injury Molecule-1 (KIM-1; Figure 5(h)).

GS-492429 treatment had a profound inhibitory effect on glomerular damage on day 14 NTN, with a 70% reduction in glomerular crescent formation

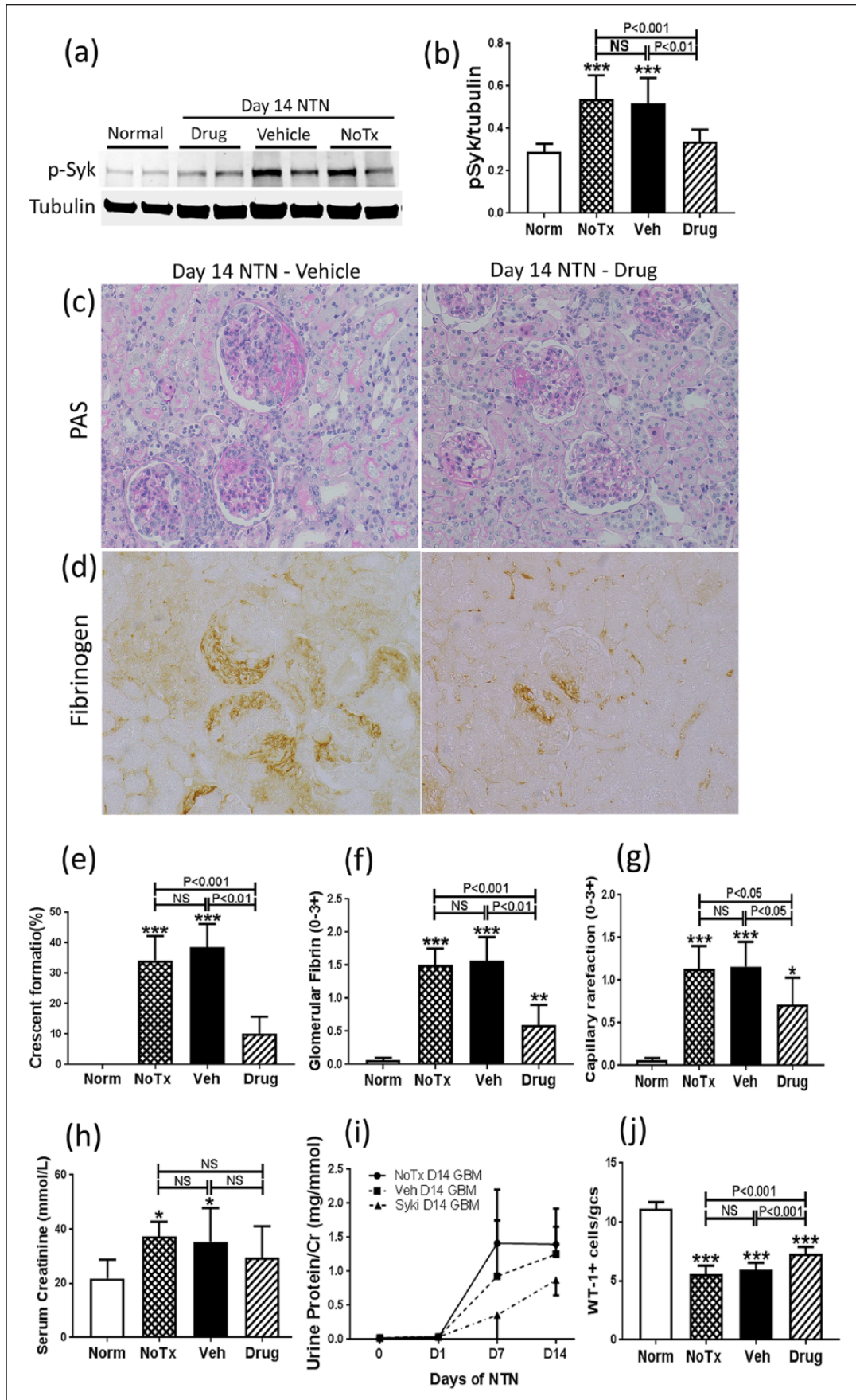


Figure 4. (Continued)

Figure 4. Effect of GS-492429 on glomerular lesions on day 14 of NTN. (a) Western blot detecting p-SYK on day 14 NTN. (b) Graph of Western blot data. (c) PAS staining of vehicle- and GS-492429-treated day 14 NTN. (d) Immunostaining for fibrinogen in vehicle and GS-492429 treatment day 14 NTN. Graphs show quantification of (e) glomerular crescent formation, (f) glomerular fibrin deposition and (g) the glomerular capillary rarefaction index. (h) Serum creatinine levels. (i) Urinary protein-to-creatinine (Cr) ratio. (j) Number of WT-1+ podocytes per glomerular cross-section (gcs). Data are mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus normal. NS: not significant. Original magnification, $\times 400$ (c and d).

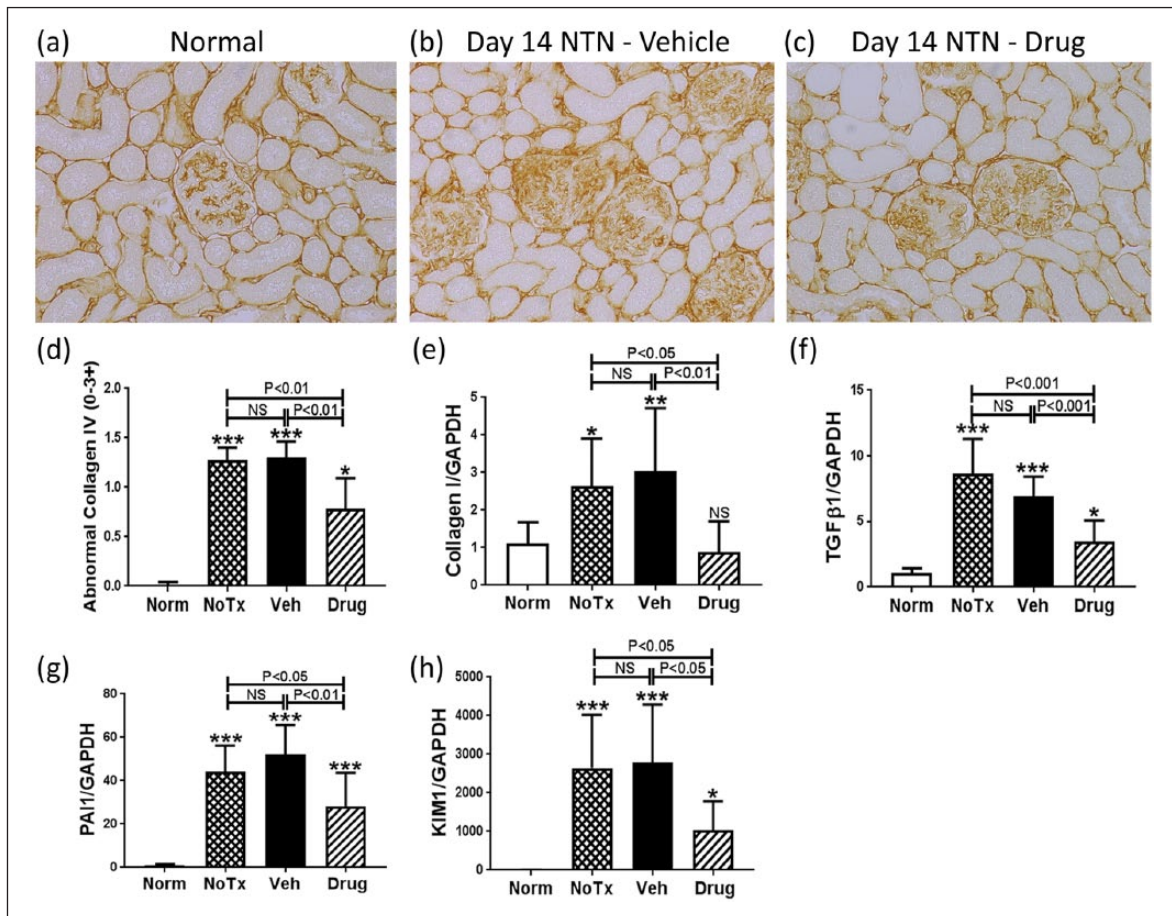


Figure 5. Renal fibrosis and tubular damage on day 14 of rat NTN. Immunostaining for collagen IV in (a) normal rat kidney, (b) untreated day 14 NTN and (c) GS-492429-treated day 14 NTN. (d) Quantification of abnormal glomerular collagen IV deposition. Analysis of mRNA levels in whole kidney tissue by real-time PCR for (e) type I collagen, (f) TGF-β1, (g) PAI-1 and (h) KIM-1. Data are mean \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus normal. Original magnification, $\times 400$ (a-c).

in conjunction with reduced glomerular fibrin deposition and glomerular capillary loss (Figure 4(c)–(g)). Drug treatment also significantly reduced glomerulosclerosis and reduced mRNA levels of collagen I, TGF-β1 and PAI-1 (Figure 5(c)–(g)). In addition, drug treatment reduced tubulointerstitial damage as shown by PAS staining and reduced KIM-1 mRNA levels (Figures 4(c) and 5(h)). However, drug treatment did not affect glomerular deposition of sheep IgG, rat IgG and C3 on day 14 of rat NTN (Supplementary Figure 1).

The untreated and vehicle-treated groups exhibited impaired renal function on day 14 NTN based on increased serum creatinine levels (Figure 4(h)). These groups also developed heavy proteinuria on days 7 and 14 NTN, with a significant reduction in the number of glomerular podocytes (Figure 4(i) and (j)). GS-492429 treatment resulted in a partial reduction in serum creatinine (Figure 4(h)). GS-492429 treatment showed a significant reduction in proteinuria on day 7 NTN, but this was not significantly lower on day 14 NTN (Figure 4(i)). However, consistent with the trend in lower

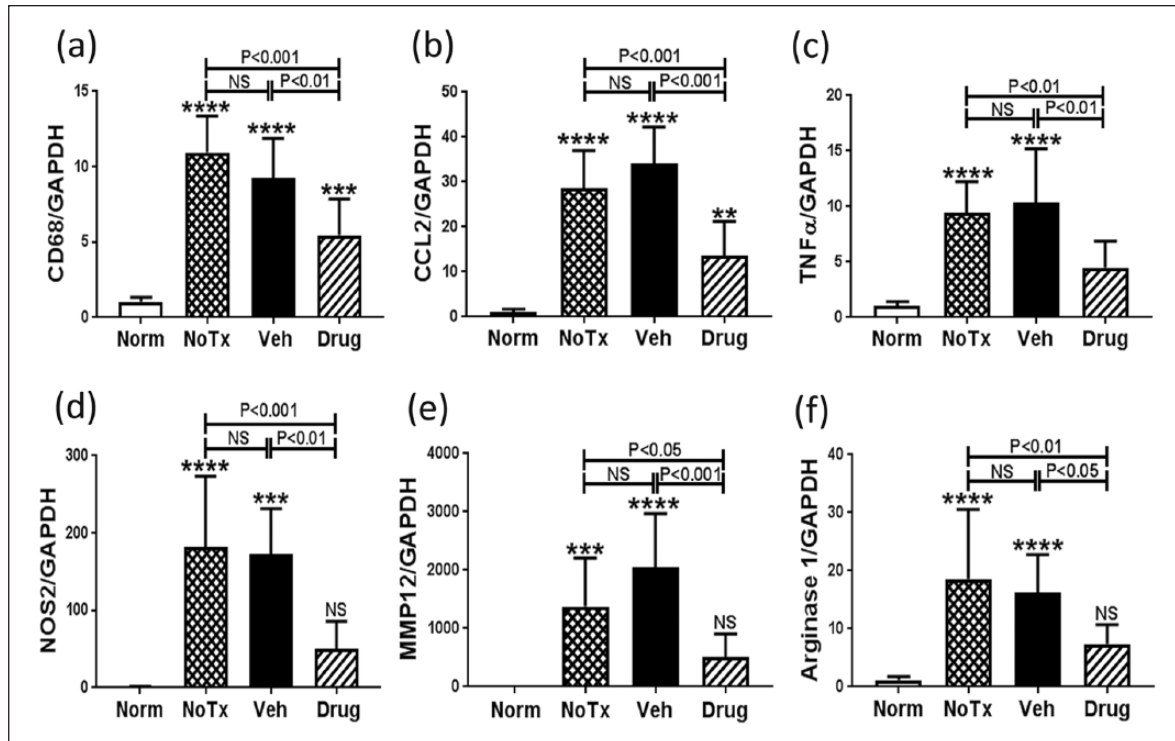


Figure 6. Macrophage infiltration and activation on day 14 of rat NTN. Real-time PCR of whole kidney samples showing mRNA levels for (a) CD68, (b) CCL2, (c) TNF- α , (d) NOS2, (e) MMP-12 and (f) arginase-1. Data are mean \pm SD. * P <0.05; ** P <0.01; *** P <0.001 versus normal.

proteinuria, GS-492429 treatment resulted in a partial protection against loss of glomerular podocytes (Figure 4(j)).

GS-492429 reduced macrophage infiltration and suppressed renal inflammation on day 14 NTN

Prominent glomerular and interstitial macrophage accumulation was evident on day 14 of untreated and vehicle-treated rat NTN demonstrated by immunostaining for CD68+ macrophages and elevated mRNA level of CD68 in whole kidney (Figures 1(e) and 6(a)). This macrophage infiltration was associated with increased mRNA levels of general markers of inflammation (CCL2 and TNF- α) as well as markers of macrophage activation (NOS2, MMP-12 and Arginase-1; Figure 6(d)–(f)). GS-492429 treatment caused a partial reduction in macrophage infiltration, along with a partial reduction in the presence of SYK+ cells, and substantially inhibited the upregulation of mRNA levels for markers of general inflammation and macrophage activation (Figures 1(g) and (h) and 6(a)–(g)).

GS-492429 did not affect T cell infiltration and activation in day 14 NTN

Immunostaining for R73+ T cells identified a substantial glomerular and interstitial T cell infiltrate on day 14 NTN in the untreated and vehicle-treated groups (Figure 7(a), (c) and (d)). RT-PCR analysis indicated infiltration of Th1 (T-bet), Th2 (GATA3), Th17 (IL-17) and regulatory (FoxP3) T cell subsets while the substantial increase in IL-2 mRNA levels indicated T cell activation (Figure 7(e)–(i)). GS-492429 treatment had no effect on glomerular or interstitial T cell infiltration (Figure 7(b)–(d)). In addition, drug treatment did not affect expression of the Th1 or Th2 markers, or T cell activation based on IL-2 mRNA levels, although FoxP3 and IL-17 mRNA levels were reduced by drug treatment (Figure 7(e)–(i)).

GS-492429 did not prevent JAK/STAT signalling

Few cells exhibited STAT3 activation (phosphorylation) in normal rat kidney (Figure 8(a)). Vehicle-treated animals exhibited small numbers of p-STAT3+ cells and were seen in glomeruli on day 1

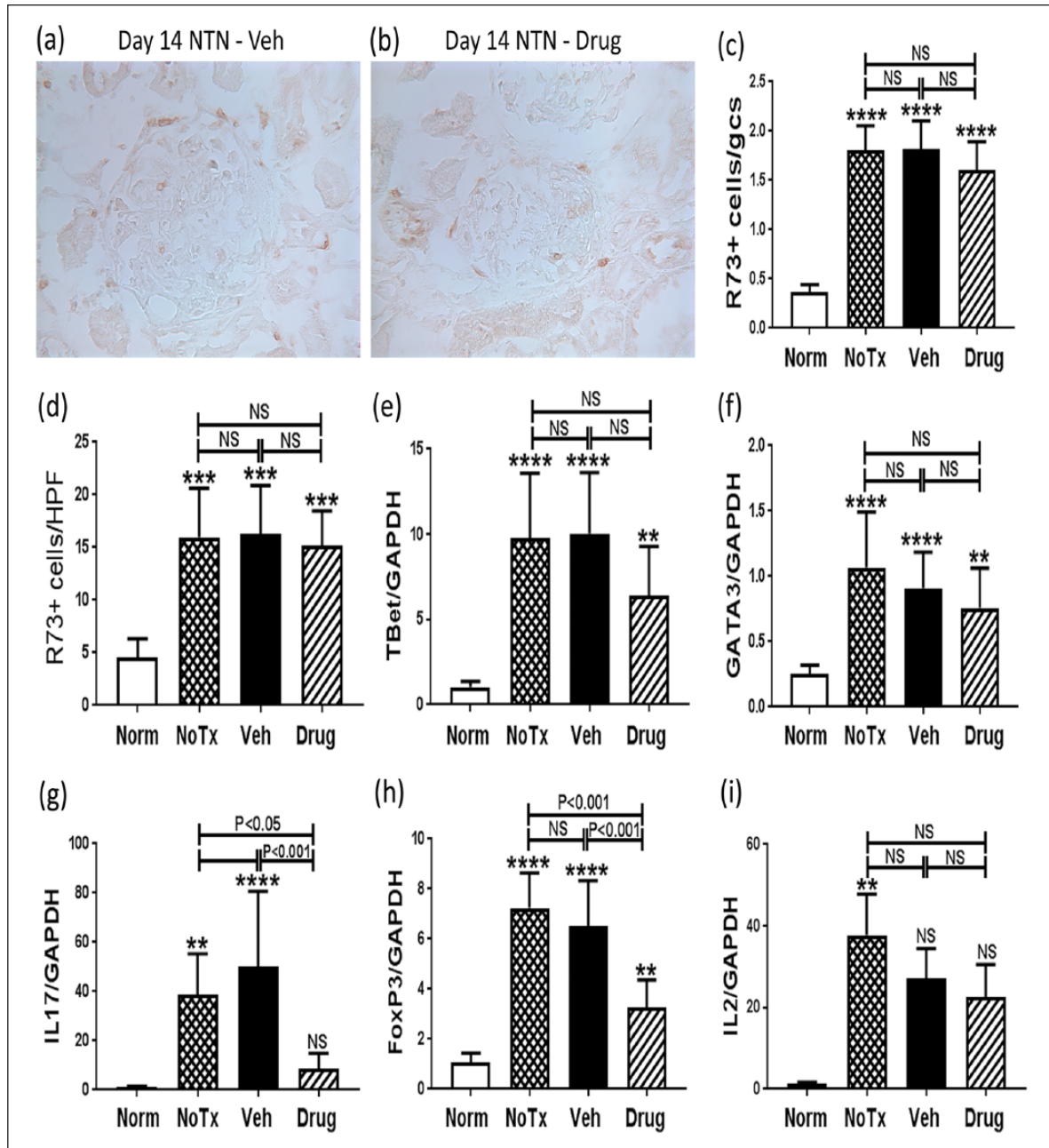


Figure 7. T cell infiltration and activation on day 14 of rat NTN. Immunostaining for R73+ T cells on (a) day 14 of rat NTN with vehicle treatment and (b) on day 14 of rat NTN with GS-492429 treatment. Graphs show quantification of T cells in the (c) glomerular and (d) interstitial compartments. Real-time PCR showing mRNA levels for (e) T-bet, (f) GATA3, (g) IL-17A, (h) FoxP3 and (i) IL-2. Data are mean \pm SD. * P < 0.05; ** P < 0.01; *** P < 0.001 versus normal.

NTN while some tubules exhibited strong nuclear p-STAT3 staining (Figure 8(b)). STAT3 phosphorylation was much more prominent of day 14 NTN, being evident in cells of the glomerular tuft and in crescents, as well as in many tubular cells (Figure 8(d) and (e)). A marked increase in p-STAT3 was also evident in Western blots of whole kidney tissue (Figure 8(g) and (h)). GS-492429 treatment did not

affect STAT3 activation on day 1 NTN (Figure 8(c)). On day 14 NTN, substantial glomerular p-STAT3 staining was evident with GS-492429 treatment, although the number of p-STAT3+ tubules was reduced (Figure 8(f)), which most probably reflects the reduction in the tubular inflammatory response. This partial reduction in STAT3 activation was also evident by Western blotting (Figure 8(g) and (h)).

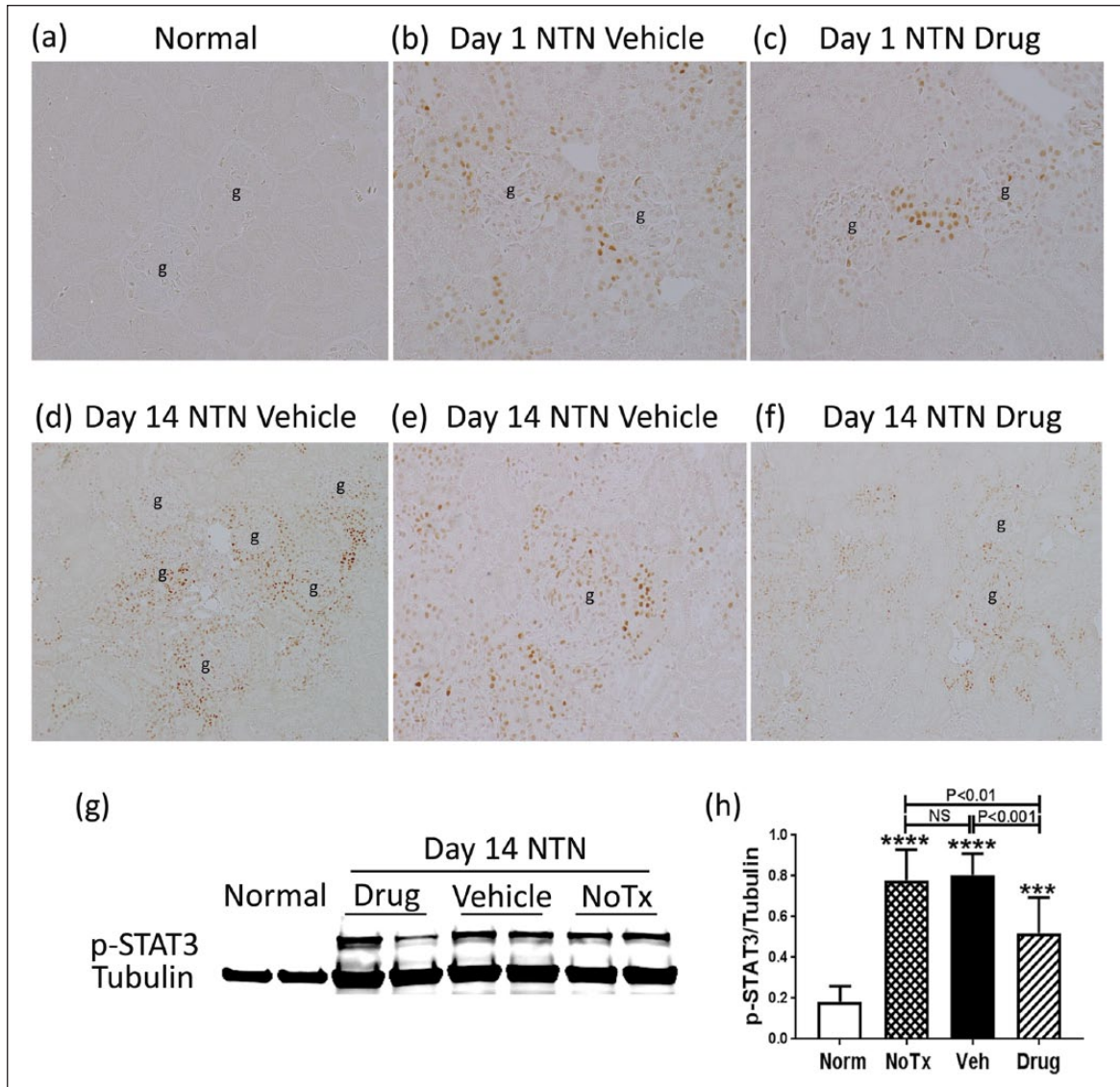


Figure 8. STAT3 activation in rat NTN. (a–f) Immunostaining for phospho-STAT3 (p-STAT3) in (a) normal rat kidney (g, glomerulus), (b) vehicle-treated day 1 NTN, (c) GS-492429-treated day 1 NTN, (d) vehicle-treated day 14 NTN, (e) cropped higher power of a glomerulus in vehicle-treated day 14 NTN and (f) GS-492429-treated day 14 NTN. (g) Western blot for p-STAT3 in day 14 NTN. (h) Graph of Western blotting data. Data are mean \pm SD. **** P < 0.001 versus normal. Original magnification: $\times 400$ (a–c, e), $\times 200$ (d and f).

Discussion

GS-492429 treatment was found to be protective in acute NTN in SD rats. This was attributed to inhibition of known mechanisms of acute glomerular injury in this model: glomerular thrombosis and glomerular infiltration of neutrophils and macrophages.^{5,7,35,37} This protection is similar to that reported in this same acute NTN model in SD rats using a different SYK inhibitor (SYKi) which has fourfold selectivity for SYK over JAK2.³⁸

GS-492429 gave substantial protection against the development of crescentic disease in the WKY rat model of NTN. This was attributed to inhibition

of glomerular thrombosis and inhibition of macrophage infiltration and macrophage M1-type pro-inflammatory activation. This is consistent with the established role of fibrin deposition and M1 pro-inflammatory macrophages, particularly macrophage MMP-12 production, in glomerular crescent formation.^{32,33,39–42} The associated reduction in glomerulosclerosis and tubular damage, events which are secondary to glomerular damage, maybe an indirect consequence of GS-492429 inhibition of glomerular damage – although a role for SYK in promoting renal interstitial fibrosis has been recently described.⁴³ Furthermore, the results

of GS-492429 treatment in rat NTN are consistent with a previous study of the mouse NTN model in which conditional *Syk* gene deletion in myeloid cells reduced acute glomerular neutrophil infiltration at 3 h and reduced macrophage accumulation and M1 pro-inflammatory activation (including MMP-12 and NOS2 expression) on day 9 NTN resulting in marked suppression of crescent formation, glomerulosclerosis and improved renal function.¹²

We investigated the question of SYK expression on day 14 rat NTN by confocal microscopy. While SYK expression in macrophages and platelets was readily apparent, we failed to demonstrate that SYK is expressed by mesangial cells or podocytes. The protection from proteinuria seen with GS-492429 treatment in the day 1 NTN model in SD rats can be attributed to inhibition of neutrophil infiltration.³⁵ However, the lack of SYK expression by podocytes may explain the limited protection of GS-492429 treatment against the development of proteinuria and podocyte loss in crescentic NTN, particularly since this model in WKY rats lacks an acute neutrophil-dependent phase of proteinuria. Indeed, *Syk* deletion in myeloid cells failed to protect against proteinuria in mouse NTN,¹² while selective macrophage depletion similarly failed to protect against the development of proteinuria in the rat NTN model despite abrogating crescent formation.³³ One limitation of our study was that we were unable to detect SYK activation in individual cell types by immunohistochemistry staining due to the currently commercially available antibodies being unsuitable for this application. However, we did identify SYK activation in the NTN model by Western blotting and this was reduced to the levels of SYK activation seen in normal kidney by GS-492429 treatment. This reduction in SYK phosphorylation could be due to one or more mechanisms. First, GS-492429 is a direct ATP-competitive inhibitor of SYK and thus would be expected to inhibit SYK phosphorylation in the disease model. Second, macrophages and platelets are the major SYK expressing cell types in this model, so that the partial reduction in both macrophage infiltration and thrombosis could account, in part, for the reduction in SYK phosphorylation with GS-492429 treatment.

GS-492429 treatment appeared to be inferior compared to the protection seen with R788 in rat NTN in WKY rats,¹⁷ although a caveat to this comparison is that the two compounds were not

compared in a side-by-side fashion with matching target coverage. In particular, R788 treatment started before administration of anti-GBM serum completely abrogated macrophage infiltration, CD8+ cell infiltration (a combination of macrophages and CD8+ T cells), crescent formation, proteinuria and renal dysfunction. This dramatic protection may relate to R406 (the active metabolite of R788) inhibiting 24 kinases more potently than SYK, including JAK2.^{19,20} There are two mechanisms by which JAK/STAT signalling contribute to the development of rat NTN. First, JAK/STAT signalling is important for T cell activation, and this model operates in a T cell-dependent fashion with macrophages as the key effectors of renal injury.^{21,23,33,40,44} Second, JAK/STAT signalling also operates in resident cells of the kidney. Indeed, conditional *Stat3* gene deletion in podocytes protects against proteinuria and crescent formation in mouse NTN,⁴⁵ and STAT3 activation in tubular epithelial cells promotes tubulointerstitial damage in a mouse model of nephron reduction.⁴⁶

In our study, we confirmed that T cells in the inflamed kidney do not express SYK and our data argue that GS-492429 did not directly affect JAK/STAT signalling; the minor reduction in tubular STAT3 phosphorylation seen with GS-492429 being attributed to an indirect effect of the overall reduction in tubular damage. GS-492429 treatment suppressed the development of rat crescentic disease without affecting T cell infiltration. This is consistent with a recent study in which GS-492429 suppressed renal injury in a rat model of acute antibody-mediated kidney allograft rejection without affecting T cell infiltration or T cell activation.⁴⁷ In addition, we identified substantial STAT3 activation in the rat NTN model, in both glomerular and, in particular, tubulointerstitial compartments. Finally, the renal injury still evident in GS-492429 treatment animals might be ameliorated by additional treatment with a JAK or STAT3 inhibitor.

In conclusion, we have established that SYK expression is restricted to myeloid cells and platelets in rat NTN and demonstrate that pharmacologic blockade of SYK signalling can suppress the development of crescentic glomerulonephritis.

Declaration of conflicting interests

J..D.P. is an employee of Gilead Sciences. The study was designed by D.J.N.-P.

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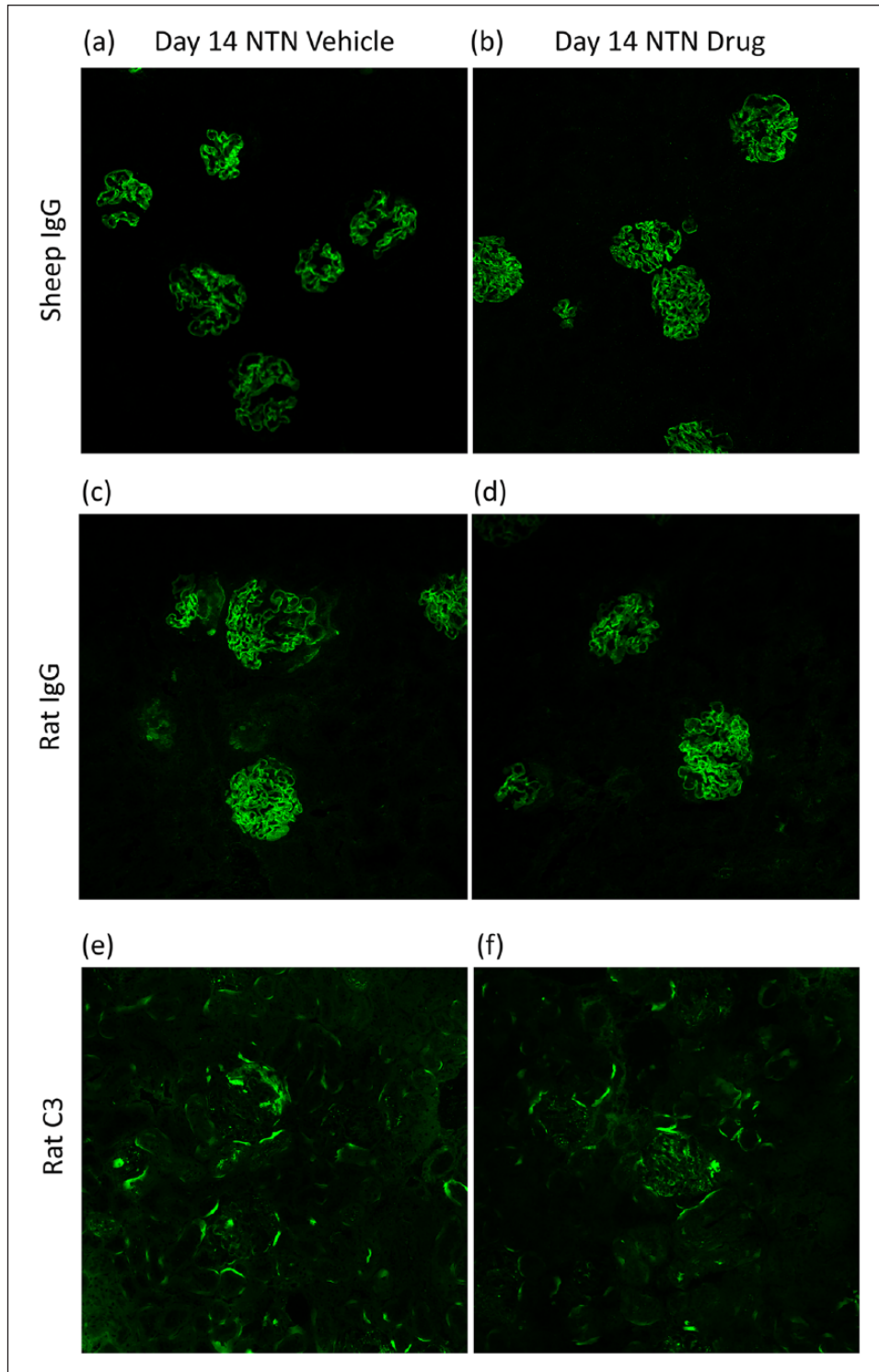
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Supplementary Table 1. A list of the top 40 hits from the primary kinome screen of 400 kinases evaluated for Kd using GS-492429 at 10 μ M.

Gene Id	Result (Kd value)
ADCK4	410
BIKE	6200
BLK	3300
CAMK1	1100
CLK3	920
CSF1R	710
CSNK2A1	40000
FAK	4900
FGR	1000
FLT3	1500
FYN	3500
GAK	400
HCK	2200
HIPK1	1100
HIPK2	780
HIPK3	940
IKK-alpha	5100
IRAK1	770
IRAK3	130
JAK2(JH1 domain-catalytic)	1300
KIT	540
LCK	2800
PDGFRB	3200
PDPK1	980
PIK4CB	1500
PIP5K1A	2900
PYK2	2500
RIOK1	40000
RIOK2	370
RIOK3	40000
SLK	3000
SRC	570
SRMS	1200
STK16	1400
SYK	9
TAK1	1100
TYK2(JH1 domain-catalytic)	3000
ULK3	1400
ZAP70	1100
YSK4	1700



Supplementary Figure 1. Effect of GS-492429 on antibody and complement deposition on day 14 of rat NTN. Confocal microscopy shows equivalent glomerular deposition of sheep IgG, rat IgG and rat C3 in vehicle treated and GS-492429 treatment day 14 NTN. Original magnification, $\times 250$.