CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Spleen Tyrosine Kinase Signaling Promotes Myeloid Cell Recruitment and Kidney Damage after Renal Ischemia/Reperfusion Injury

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Ischemia/reperfusion (I/R) injury is an important cause of acute and chronic renal failure. Neutrophils and macrophages, by integrin-based recruitment, play a key role in renal I/R injury. Integrin-based activation of spleen tyrosine kinase (Syk) contributes to myeloid cell adhesion to activated endothelial cells *in vitro*; however, whether Syk is required for myeloid cell recruitment and tubular damage in I/R injury is unknown. Therefore, we investigated the function of Syk in mouse I/R injury using two different approaches. C57BL/6J mice underwent bilateral warm ischemia and were sacrificed after 30 minutes or 24 hours of reperfusion. Mice were treated with the Syk inhibitor CC0417, or vehicle, beginning 1 hour before surgery. Syk was expressed by infiltrating neutrophils, macrophages, and platelets in vehicle-treated I/R injury which exhibited severe renal failure and tubular damage at 24 hours. CC0417 treatment markedly reduced neutrophil, macrophage, and platelet accumulation with improved renal function and reduced tubular damage. Next, we compared mice with conditional Syk gene deletion in myeloid cells (*Syk^{My}*) versus *Syk^{f/f}* littermate controls in a 24-hour study. *Syk^{My}* mice also showed a marked reduction in neutrophil and macrophage infiltration with significant protection from I/R-induced acute renal failure and tubular damage. These studies define a pathologic role for myeloid Syk signaling in renal I/R injury and identify Syk as a potential therapeutic target in this condition. (*Am J Pathol* 2016, 186: 2032–2042; <http://dx.doi.org/10.1016/j.ajpath.2016.04.007>)

Renal ischemia/reperfusion (I/R) injury is a major clinical problem with significant morbidity and mortality. It is the leading cause of acute renal failure after renal transplantation, shock, sepsis, and renal artery stenosis. Renal I/R injury in transplantation is associated with delayed graft function and is one of the major risk factors for acute renal transplant rejection. In addition, acute I/R injury also greatly increases the risk of development of chronic rejection and other chronic renal diseases.^{1–3} Current therapies for renal I/R injury are still only supportive. Therefore, it is important to gain knowledge of the underlying mechanisms and to try to identify potential therapeutic targets.

The pathogenesis of renal I/R injury is complex and involves both direct damage to tubular epithelial cells through hypoxia/re-oxygenation and ATP depletion, as well as indirect tubular damage through the recruitment and activation of neutrophils, monocyte/macrophages, and platelets.^{4–6} Although the

participation of leukocytes and platelets in I/R injury is well established, we have only a limited understanding of the signaling events involved in this key pathologic process.

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase strongly up-regulated on I/R injury in the murine kidney.⁷ It is expressed by most leukocyte populations, except mature T cells, as well as by some nonhematopoietic lineage

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cells.^{8,9} Syk is best known for its role downstream of immune receptors to trigger a series of signaling pathways leading to proliferation, survival, differentiation, migration, and production of reactive oxygen species and cytokines.⁹ Syk has been shown to promote antibody-associated glomerulonephritis and rheumatoid arthritis injury by facilitating activation of neutrophils, macrophages, and platelets.^{10–16} The protective effects of nonselective kinase inhibitors in models of I/R injury in retina, brain, and intestine suggest that Syk may be involved in this response.^{17–20} However, a role for Syk in I/R injury remains to be established.

Our aim was to determine whether Syk signaling plays a pathologic role in renal I/R injury using both genetic and pharmaceutical approaches.

Materials and Methods

Antibodies

The following antibodies were used in this study: rat anti-mouse CD68 and fluorescein isothiocyanate-conjugated F4/80 (Serotec, Oxford, UK) both of which recognize macrophages; unconjugated and allophycocyanin-conjugated rat anti-mouse GR1 that recognize neutrophils (BioLegend, San Diego, CA); rat anti-mouse CD41 that recognizes activated platelets (Serotec); rabbit anti-fibrinogen γ (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Syk (Cell Signaling, Danvers, MA), and mouse antitubulin (Sigma-Aldrich, Castle Hill, NSW, Australia). Secondary antibodies for immunofluorescence staining included fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG, fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (Serotec), and allophycocyanin-conjugated goat anti-rabbit IgG (Invitrogen, Eugene, OR). The biotinylated secondary antibody was goat anti-rabbit IgG (Invitrogen) which was detected with an avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). Other secondary detection systems included horseradish peroxidase-conjugated rabbit anti-rat IgG and rat peroxidase-conjugated anti-peroxidase complexes (Dako, Glostrup, Denmark).

Syk Inhibitor CC0482417

CC0482417 (CC0417) was manufactured by Celgene (San Diego, CA). CC0417 inhibits Syk enzyme activity with a concentration that inhibits 50% (IC_{50}) of 3.1 nmol/L. In a panel of 71 enzymes, the closest enzymes inhibited were Janus kinase 2 (IC_{50} , 15.9 nmol/L), Janus kinase 1 (IC_{50} , 16.5 nmol/L), and Janus kinase 3 (IC_{50} , 34.7 nmol/L). CC0417 has no activity against ζ -associated protein 70.²¹ CC0417 was prepared in 20% hydroxylpropyl- β -cyclodextrin vehicle (Sigma-Aldrich) and administered by twice daily gavage at 30 mg/kg as previously described.²¹

Mouse Model of Renal I/R Injury

Male C57BL/6J mice were obtained from Monash Animal Research Platform (Clayton, VIC, Australia). *Syk^{fl/fl}* mice

were a kind gift from Professor Alexander Tarakhovsky (The Rockefeller University, New York, NY)²² and were backcrossed onto the C57BL/6J background for eight generations. *Syk^{fl/fl}* mice were then crossed with *LysM^{Cre}* mice (C57BL/6J) to produce *Syk^{My}* mice that have Syk deletion in neutrophils and macrophages as previously described.²³ All mice used were male and aged between 9 to 12 weeks.

Mice were anesthetized with ketamine/xylazine and then placed on a heating blanket with a rectal temperature probe to maintain body temperature at 37°C. After abdominal incision, both renal pedicles were clamped using non-traumatic vascular clamps. The abdomen was closed with temporary sutures to reduce fluid and heat loss. After ischemia, clamps were removed, reperfusion of the kidneys was visually confirmed, and then animals were fully sutured. Mice were given intraperitoneal normal saline post-operatively. Pilot studies investigating periods of 15 to 22 minutes of warm ischemia were performed and identified that ischemic periods of 17 and 19 minutes induced reliable and severe acute kidney injury at 24 hours without causing loss of animals.

In the first experiment, groups of C57BL/6J mice ($n = 8$) underwent I/R surgery (19 minutes of ischemia) and were treated twice daily with 30 mg/kg Syk inhibitor CC0417 or vehicle, given by oral gavage, beginning 1 hour before surgery. Animals were euthanized 24 hours after reperfusion. To investigate the early events after reperfusion, groups of mice ($n = 8$) treated with CC0417 or vehicle were sacrificed 30 minutes after reperfusion. Sham-operated mice were used as controls.

In the second experiment, *Syk^{My}* ($n = 8$) or *Syk^{fl/fl}* ($n = 8$) mice underwent I/R surgery (17 minutes of ischemia) as described earlier in this section and sacrificed after 24 hours. Sham-operated animals were used as controls. All animal experiments were approved by Monash Medical Center Animal Ethics Committee.

Histologic Examination

Periodic acid-Schiff staining was performed on 2- μ m sections of formalin-fixed tissue. The outer medulla of the right kidney from each animal was examined under high power ($\times 400$). Tubular damage was defined as tubular swelling (gross enlargement and partial detachment from the tubular basement membrane), loss of the brush border, or nuclear loss and expressed as the percentage of tubules exhibiting damage. All analysis was performed on blinded slides (J.R. and F.Y.M.).

Immunohistochemistry

Immunoperoxidase staining for fibrinogen was performed on formalin-fixed sections with antigen retrieval (citrate buffer, pH 6.0) using an avidin-biotin complex method. Immunostaining for Syk, neutrophils, macrophages, and

platelets was performed on paraformaldehyde-fixed cryostat sections using a peroxidase-conjugated anti-peroxidase method. Two color immunofluorescence staining for Syk, macrophages, neutrophils, and platelets was performed on paraformaldehyde-fixed cryostat sections using a Nikon C1 confocal microscope (Nikon, Melville, NY).

The number of interstitial CD68⁺ macrophages and GR-1⁺ neutrophils were counted in at least 20 high-power fields ($\times 400$) in the outer medulla in each animal and expressed as cells per mm². Immunostaining for fibrinogen and CD41⁺ platelets was assessed as positively stained clumps per mm² under high-power ($\times 400$) in the outer medulla or as area stained using ImageJ software version 1.41 (NIH, Bethesda, MD).²⁴

Real-Time RT-PCR

Real-time PCR was performed as described previously.^{25–27} Mouse kidney cross-section samples were snap-frozen in liquid nitrogen and stored at -80°C until RNA was extracted using a RiboPure RNA isolation kit (Ambion, Austin, TX). cDNA was prepared from total RNA by reverse transcription using random hexamer primers (Invitrogen). Real-time PCR was performed on a StepOne machine (Applied Biosystems, Scoresby, VIC, Australia) with thermal cycling conditions of 37°C for 10 minutes, 95°C for 5 minutes, followed by 50 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 68°C for 20 seconds. The primer pairs and FAM-labeled minor groove binder probes were

Figure 1 Expression of Syk in mouse I/R kidney. **A:** Immunoperoxidase staining for Syk in sham-operated and in vehicle- and CC0417-treated animals at 24 hours after reperfusion. **B:** Two-color confocal images showing colocalization of Gr-1⁺ neutrophils (green) with Syk⁺ cells (red) in I/R kidney with double-stained cells indicated as yellow (arrows) in the merged image. **C:** Two-color confocal images showing colocalization of F4/80⁺ macrophages (green) with Syk⁺ cells (red) in I/R kidney with double-stained cells indicated in yellow (arrows). **D:** Two-color confocal images showing colocalization of CD41⁺ platelets (green) with Syk (red) in I/R kidney with double-stained cells shown in yellow (arrows). **B–D:** Sections are counterstained with DAPI (blue). Original magnification, $\times 250$ (**A–D**). APC, allophycocyanin; FITC, fluorescein isothiocyanate; I/R, ischemia/reperfusion; SYK, spleen tyrosine kinase; Veh, vehicle.

Figure 2 Effect of CC0417 on histologic damage at 24 hours after renal I/R injury. **A–F:** Periodic acid-Schiff–stained sections of sham-operated (**A** and **B**), Veh-treated I/R (**C** and **D**), and CC0417-treated (**E** and **F**) I/R. **G:** Quantification of the percentage of injured tubules in the outer medulla. **H:** Real-time RT-PCR analysis of KIM-1 mRNA levels. Data are expressed as means ± SD. $n = 8$ (**G** and **H**). *** $P < 0.001$ (one-way analysis of variance with Bonferroni's multiple-comparison test). Original magnification: $\times 250$ (**A**, **C**, and **E**); $\times 400$ (**B**, **D**, and **F**). I/R, ischemia/reperfusion; KIM-1, kidney injury molecule 1; Veh, vehicle.

obtained from Applied Biosystems or designed as previously described.^{25–27} The relative amount of mRNA was calculated using the comparative Ct method. All specific amplicons were normalized against 18S rRNA, which was amplified in the same reaction as an internal control using commercial assay reagents (Applied Biosystems).

Statistical Analysis

Data are expressed as means ± SD. Results were analyzed using parametric analysis of variance with post hoc analysis, using Bonferroni's post-test for multiple comparisons or

with an unpaired *t*-test with Welch's correction. Analyses were performed with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA).

Results

Syk Expression in Renal I/R Injury

Immunostaining showed only a small number of Syk⁺ cells in the sham-operated kidney which appeared to be mainly leukocytes (Figure 1A). In contrast, prominent Syk staining was evident in the kidney at 24 hours in vehicle-treated mice. These Syk⁺ cells were predominantly localized to the outer medulla, and Syk staining was restricted to the interstitial space (Figure 1A). Two-color immunostaining showed that Syk was expressed by infiltrating neutrophils, macrophages, and CD41⁺ activated platelets in vehicle-treated I/R kidney (Figure 1, B–D).

CC0417 Treatment Suppresses Renal I/R Injury

Vehicle-treated animals developed severe tubular necrosis in the outer medulla at 24 hours after I/R injury (Figure 2,

Figure 3 Effect of CC0417 on renal function, leukocyte infiltration, and platelet activation at 24 hours after renal I/R injury. **A** and **B:** Renal function as measured by serum creatinine (**A**) and BUN (**B**). **C–F:** Graphs showing quantification of immunostaining of tissue sections for Gr-1⁺ neutrophils (**C**); CD68⁺ macrophages (**D**); CD41⁺ platelets (**E**), and fibrinogen (**F**). Data are expressed as means ± SD. $n = 8$ (**A–F**). ** $P < 0.01$, *** $P < 0.001$ (one-way analysis of variance with Bonferroni's multiple-comparison test). BUN, blood urea nitrogen; I/R, ischemia/reperfusion; Veh, vehicle.

A–D). These histologic changes were evident in approximately 60% of tubules (Figure 2G) and were associated with a massive up-regulation of kidney injury molecule 1 (KIM-1) mRNA (Figure 2H). Consistent with the histologic examination, vehicle-treated mice developed severe renal impairment with a 17-fold increased serum creatinine concentration and an eightfold increased blood urea nitrogen concentration (Figure 3, A and B). In contrast, treatment with CC0417 substantially reduced the severity of tubular necrosis as shown by histology and KIM-1 mRNA levels (Figure 2, E–H). This protective effect was associated with a significant improvement in renal function (Figure 3, A and B).

CC0417 Treatment Reduces Leukocyte Recruitment and Platelet Activation in Renal I/R Injury

Vehicle-treated mice featured prominent infiltration of neutrophils and macrophages, as well as platelet activation and fibrin deposition, at 24 hours of I/R injury (Figure 3, C–F). In contrast, CC0417 treatment markedly suppressed the accumulation of Syk⁺ cells at 24 hours after I/R injury (Figure 1A and Figure 3, C–F). This was mirrored by a substantial reduction in neutrophil and macrophage infiltration, as well as platelet aggregation and fibrin deposition, at 24 hours in renal I/R injury (Figure 3, C–F).

To determine whether CC0417 treatment prevented the early leukocyte infiltrate and platelet activation, groups of mice were sacrificed after 30 minutes of reperfusion. A

significant increase in neutrophils, macrophages, and prominent platelet deposition was evident in vehicle-treated mice at 30 minutes after I/R injury. CC0417 treatment largely abrogated neutrophil infiltration and platelet accumulation at 30 minutes after I/R injury, but it did not affect the minor macrophage infiltration (Figure 4).

CC0417 Treatment Ameliorates Inflammation in Renal I/R Injury

Analysis of leukocyte and inflammation markers was performed by real-time PCR in whole kidney tissue at 24 hours after I/R injury. CC0417 treatment significantly reduced CD68 mRNA levels (Figure 5A), consistent with immunohistochemistry data for CD68⁺ macrophages. This reduction in macrophage infiltration was associated with a reduction in the monocyte chemokine monocyte chemoattractant protein 1 (Figure 5B).

Markers of proinflammatory M1-type macrophages (nitric oxide synthase 2 and metalloproteinase 9 and 12) and M2-type alternatively activated macrophages (arginase-1, CD163, and CD206) were increased in vehicle-treated renal I/R injury (Figure 5, C–H). Activation of the inflammatory response in vehicle-treated I/R injury was also evident with up-regulation of mRNA levels of tumor necrosis factor- α , IL-6, IL-1 β , IL-18, and NLR family, pyrin domain containing 3 (NLRP3) (Figure 5, I–M). CC0417 treatment significantly reduced mRNA levels of markers of both M1 and M2 type macrophages (Figure 5, C–H) and reduced

Figure 4 Effect of CC0417 on leukocyte infiltration and platelet activation at 30 minutes after renal I/R injury. **A** and **B**: Immunostaining for Gr-1⁺ neutrophils in vehicle-treated (**A**) and CC0417-treated (**B**) renal I/R injury. Quantification of Gr-1⁺ neutrophils. **C**–**E**: Immunostaining for CD68⁺ macrophages (**D**) in vehicle-treated (**D**) and CC0417-treated (**E**) renal I/R injury. **F**: Quantification of CD68⁺ macrophages. **G** and **H**: Immunostaining for CD41⁺ platelets in vehicle-treated (**G**) and CC0417-treated (**H**) renal I/R injury. **I**: Quantification of CD41⁺ platelets. Data are expressed as means $\pm$ SD. $n = 8$. * $P < 0.05$ (one-way analysis of variance with Bonferroni's multiple-comparison test). Original magnification: $\times 400$ (**A**, **B**, **G**, and **H**); $\times 250$ (**D** and **E**). I/R, ischemia/reperfusion; Veh, vehicle.

Figure 5 Effect of CC0417 on renal inflammation at 24 hours after renal I/R injury. Real-time RT-PCR analysis of whole-kidney tissue for mRNA levels of CD68 (A), MCP-1 (B), NOS2 (C), MMP-12 (D), MMP-9 (E), (ARG1) (F), CD163 (G), CD206 (H), TNF-α (I), IL-6 (J), IL-1β (K), IL-18 (L), NLRP3 (M), and HO-1 (N). Data are expressed as means ± SD. $n = 8$ (A–N). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-way analysis of variance with Bonferroni's multiple-comparison test). ARG1, arginase-1; HO-1, heme oxygenase-1; I/R, ischemia/reperfusion; MCP-1, monocyte chemoattractant protein 1; MMP, metalloproteinase; NLRP3, NLR family, pyrin domain containing 3; NOS2, nitric oxide synthase 2; TNF, tumor necrosis factor; Veh, vehicle.

expression of the inflammatory response, except for IL-1β (Figure 5, I–M). However, CC0417 treatment had no effect on the up-regulation of heme oxygenase 1 (Figure 5N), which plays a protective role in I/R injury.²⁸

Syk Gene Deletion in Myeloid Cells Ameliorates Renal I/R Injury

To confirm the selectivity of the kinase inhibitor study and to define the specific contribution of Syk signaling in myeloid cells, we used mice with conditional Syk gene deletion. *Syk^{fl/fl}LysM^{Cre}* (abbreviated to *Syk^{My}*) mice exhibit Syk deletion in most blood neutrophils and monocytes.²³ These mice are healthy and have normal white blood cell counts and resident macrophage populations.²³

Control *Syk^{fl/fl}* mice developed severe tubular necrosis after renal I/R injury as illustrated by histologic examination and KIM-1 mRNA levels, and this was associated with severe renal failure (Figure 6, A, C–F). By contrast, *Syk^{My}* mice showed a substantial improvement in renal function and diminished tubular damage, including reduced KIM-1 mRNA levels, in response to I/R injury (Figure 6, B–F).

Renal I/R injury induced a prominent infiltrate of Syk⁺ cells in the outer medulla in control *Syk^{fl/fl}* mice (Figure 6, G–I). This was coincident with infiltration of neutrophils, macrophages, and platelet activation in *Syk^{fl/fl}* mice (Figure 7). However, this infiltration of neutrophils and macrophages was substantially reduced in *Syk^{My}* mice, whereas platelet activation showed a nonsignificant trend

toward a reduction, and fibrin deposition was reduced in *Syk^{My}* mice (Figure 7).

The reduction in CD68⁺ macrophages seen in *Syk^{My}* mice after I/R injury was confirmed by PCR analysis of whole kidney CD68 mRNA levels, and this was associated with reduced monocyte chemoattractant protein 1 expression (Figure 8, A and B). PCR analysis showed significant increase in expression of markers of M1 and M2 type macrophages and activation of the inflammatory response after I/R injury in *Syk^{fl/fl}* mice (Figure 8, C–M). By contrast, the up-regulation of M1 and M2 markers and activation of the inflammatory response was significantly reduced in *Syk^{My}* mice, except for IL-1β (Figure 8, C–M). However, up-regulation of heme oxygenase 1 was not affected in *Syk^{My}* mice (Figure 8N).

Discussion

Our data provide clear evidence that Syk in myeloid cells is required for renal I/R injury. Myeloid-specific Syk gene deletion profoundly suppressed neutrophil and macrophage recruitment into the kidney and consequent renal injury. This finding was recapitulated by systemic treatment with the Syk inhibitor CC0417.

Syk is expressed by most leukocytes, except mature T cells, and by platelets.^{8,9} Two-color staining identified neutrophils, macrophages, and platelets as the major populations of Syk-expressing cells in the kidney after I/R injury. Syk expression by various nonleukocyte cell types

Figure 6 Effect of conditional Syk deletion in myeloid cells (Syk^{My}) compared with control $Syk^{fl/fl}$ mice on histologic damage and renal function at 24 hours after renal I/R injury. **A** and **B**: Periodic acid-Schiff–stained sections of Syk^{My} (**A**) and $Syk^{fl/fl}$ (**B**) mice after I/R injury. **C**: Graphs showing the percentage of injured tubules in outer medulla. **D**: RT-PCR analysis of KIM-1 mRNA levels in $Syk^{fl/fl}$ (white bar) and Syk^{My} (black bar) mice. **E** and **F**: Renal function as measured by serum creatinine (**E**) and BUN (**F**). **G–I**: Immunoperoxidase staining for Syk in $Syk^{fl/fl}$ sham operated (**G**), $Syk^{fl/fl}$ I/R injury (**H**), and Syk^{My} I/R injury (**I**). Data are expressed as means $\pm$ SD. $n = 8$. * $P < 0.05$, ** $P < 0.01$ (one-way analysis of variance with Bonferroni's multiple-comparison test). Original magnification: $\times 250$ (**A**, **B**, and **G–I**). BUN, blood urea nitrogen; I/R, ischemia/reperfusion; KIM-1, kidney injury molecule 1; SYK, spleen tyrosine kinase.

has also been reported, including mesangial cells, fibroblasts, smooth muscle cells, and tubular epithelial cells,^{8,9} but we did not identify Syk expression in these cell types after renal I/R injury. This clear increase in Syk-expressing cells is consistent with a previous PCR-based study that identified a significant increase in Syk mRNA after renal I/R

injury.⁷ However, one limitation of the present study was our inability to detect active (phosphorylation) Syk by immunohistochemistry in mouse tissue sections because of a lack of suitable commercial antibodies.

Both systemic CC0417 treatment and myeloid-specific Syk gene deletion profoundly suppressed neutrophil and macrophage recruitment into the kidney at 30 minutes and 24 hours after I/R injury. Our findings are consistent with the known role of myeloid cells in exacerbating renal I/R injury.^{6,29} It is well established that neutrophils attach to the disrupted endothelium and accumulate in the peritubular capillary network of the outer medulla as early as 30 minutes after reperfusion.^{29–31} The blockade of leukocyte endothelial adhesion molecules, including $\beta 2$ -integrins,³² intercellular adhesion molecule 1,^{29,33} E-selectin,³⁴ and P-selectin,³⁵ is protective in models of renal I/R injury. Importantly, Syk signaling can be activated after integrin/ligand interactions through a transmembrane adaptor, such as DNAX activation protein of 12 kDa or the FcR common γ -chain.^{36,37} The mechanism underlying the blockade of myeloid cell recruitment in the present study is likely to involve Syk signaling in integrin-based myeloid cell recruitment on the basis that Syk is required for integrin-based adhesion of neutrophils to activate endothelial cells in culture³⁸ and that chemotaxis of myeloid cells *in vitro* involves a Syk/integrin-dependent mechanism.^{39,40} However, not all neutrophil recruitment at sites of inflammation operates by a Syk-dependent mechanism,⁴¹ indicating selectivity in this process.

Platelet activation exacerbates renal I/R injury.⁴² A role for Syk in platelet activation by Fc receptor has been

Figure 7 Effect of conditional Syk deletion in myeloid cells (Syk^{My}) compared with control $Syk^{fl/fl}$ mice on cell infiltration at 24 hours after renal I/R injury. **A–D**: Graphs show Gr-1⁺ neutrophils (**A**), CD68⁺ macrophages (**B**), deposition of CD41⁺ platelets (**C**), and deposition of fibrinogen (**D**) in $Syk^{fl/fl}$ (white bar) and Syk^{My} (black bar) mice. Data are expressed as means $\pm$ SD. $n = 8$. * $P < 0.05$ (one-way analysis of variance with Bonferroni's multiple-comparison test). I/R, ischemia/reperfusion; SKY, spleen tyrosine kinase.

Figure 8 Effect of conditional Syk deletion in myeloid cells (*Syk^{My}*) compared with control *Syk^{fl/fl}* mice on renal inflammation at 24 hours after renal I/R injury. Real-time RT-PCR analysis of whole kidney for mRNA levels of CD68 (A), MCP-1 (B), NOS2 (C), MMP-12 (D), MMP-9 (E), ARG1 (F), CD163 (G), CD206 (H), TNF-α (I), IL-6 (J), IL-1β (K), IL-18 (L), NLRP3 (M), and HO-1 (N). Data are expressed as means ± SD. $n = 8$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-way analysis of variance with Bonferroni's multiple-comparison test). ARG1, arginase-1; HO-1, heme oxygenase-1; I/R, ischemia/reperfusion; MCP-1, monocyte chemoattractant protein 1; MMP, metalloproteinase; NLRP3, NLR family, pyrin domain containing 3; NOS2, nitric oxide synthase 2; TNF, tumor necrosis factor; Veh, vehicle.

previously described in myocardial I/R injury.^{43,44} The ability of CC0417 to inhibit platelet activation and fibrin deposition is likely to be one of the protective actions of drug treatment and is consistent with previous studies in which a Syk inhibitor suppressed platelet activation and thrombosis in a model of acute antibody-dependent glomerular injury.¹¹ Platelets may contribute to the accumulation of leukocytes in I/R tissue.⁴⁵ However, neutrophils and macrophages also play an important role in platelet activation and thrombosis, leading to fibrin deposition.^{46,47} In *Syk^{My}* mice, the reduction in fibrin deposition and the trend toward a reduction in platelet activation in renal I/R injury may be due to the reduction in myeloid cell accumulation and activation.

IL-6 is produced rapidly in response to infection and tissue injury, including in renal I/R injury.⁴⁸ Both genetic and pharmacologic inhibition of IL-6 is protective in renal I/R injury, which is associated with a substantial reduction in neutrophil infiltration and expression of tumor necrosis factor-α.⁴⁹ Our findings of a substantial reduction in IL-6 mRNA levels, together with a reduction in myeloid cell recruitment and tumor necrosis factor-α mRNA levels, are consistent with a pathologic role for IL-6 in this form of acute renal injury.

Activation of the NLRP3-associated inflammasome by the presence of necrotic cells has been implicated as a mechanism of sterile inflammation in renal I/R injury.⁵⁰ Although Syk signaling is required for activation of the

Nlrp3 inflammasome in myeloid cells, this function is largely related to the process of phagocytosis, and any involvement of Syk in inflammasome activation in renal I/R injury remains to be determined. Our finding that NLRP3 and IL-18 mRNA levels were significantly reduced with genetic or pharmacologic blockade of Syk provides circumstantial evidence to support a role for Syk in inflammasome activation in renal I/R injury; however, this finding could also simply relate to the reduction in myeloid cell recruitment.

Previous studies have examined Syk in models of I/R injury, using nonselective kinase inhibitors.^{17,18,20} Piceatannol is a naturally occurring compound found in grapes, which is often described as a selective Syk inhibitor, although it is also a potent inhibitor of ζ-associated protein 70, phosphatidylinositol 3-kinase, cyclooxygenase-2, mitochondrial proton F0F1-ATPase/ATP synthase, and p56^{lck}.^{51–54} Piceatannol treatment reduced the severity of tissue injury in models of retinal I/R injury¹⁸ and ischemic stroke.¹⁷ R788, also known as fostamatinib, inhibits a wide range of kinases, including Syk.⁵⁵ R788 treatment inhibited both local intestinal I/R injury and remote lung injury with a significant reduction in neutrophil infiltration.²⁰ However, it is unclear whether the beneficial effects of either of these inhibitors in I/R injury is actually due to targeting Syk. To our knowledge, our study is the first to delineate the role of Syk in I/R injury using a conditional gene deletion approach. The excellent concordance between the results

with myeloid cell *Syk* gene deletion and CC0417 treatment provides a strong argument that the beneficial effects of CC0417 are most likely due to *Syk* inhibition.

Syk inhibitor drugs have been used in clinical trials for multiple indications, including leukemia,^{56,57} arthritis,^{58,59} idiopathic thrombocytopenic purpura,⁶⁰ and allergic rhinitis,⁶¹ demonstrating the general tolerability of these compounds. The results of the present study establish the therapeutic potential of *Syk* inhibitors for situations in which renal I/R injury can be anticipated. Indeed, a case for *Syk* inhibition as a prophylactic treatment in patients undergoing renal allograft transplantation can be made based on the following: i) results of the present study, showing benefit of *Syk* inhibition in anticipated renal I/R injury; ii) the recently identified role for *Syk* in acute antibody-mediated renal allograft rejection²¹; and iii) the potential for *Syk* inhibition to suppress *de novo* donor-specific antibody production.⁶²

Conclusions

We have identified a pathologic role for myeloid cell *Syk* signaling in renal I/R injury using two complementary approaches. These findings identify *Syk* as a potential therapeutic target in acute ischemic kidney injury.

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