INTERNATIONAL JOURNAL OF Experimental Pathology

Int. J. Exp. Path. (2015), 96, 54–62

ORIGINAL ARTICLE

Spleen tyrosine kinase contributes to acute renal allograft rejection in the rat

Sharmila Ramessur Chandran*,†, Greg H. Tesch*,†, Yingjie Han*,†, Naomi Woodman*, William R. Mulley*,†, John Kanellis*,†, Kate Blease‡, Frank Y. Ma*,†,[1] and David J. Nikolic-Paterson*,†,[1]

**Department of Nephrology, Monash Medical Centre, Clayton, Vic., Australia, †Centre for Inflammatory Diseases, Monash Medical Centre, Monash University, Clayton, Vic., Australia and ‡Celgene, San Diego, CA, USA*

INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

doi: 10.1111/iep.12110

Received for publication: 15 October 2014
Accepted for publication: 30 October 2014

Correspondence:
David J Nikolic-Paterson
Department of Nephrology
Monash Medical Centre
246 Clayton Road, Clayton
Victoria 3168, Australia
Tel.: +61 3 95943535
Fax: +61 3 95946530
E-mail: david.nikolic-paterson@monash.edu

[1]These authors contributed equally to this study.

SUMMARY

Kidney allografts induce strong T-cell and antibody responses which mediate acute rejection. Spleen tyrosine kinase (Syk) is expressed by most leucocytes, except mature T cells, and is involved in intracellular signalling following activation of the Fcγ-receptor, B-cell receptor and some integrins. A role for Syk signalling has been established in antibody-dependent native kidney disease, but little is known of Syk in acute renal allograft rejection. Sprague–Dawley rats underwent bilateral nephrectomy and received an orthotopic Wistar renal allograft. Recipient rats were treated with a Syk inhibitor (CC0482417, 30 mg/kg/bid), or vehicle, from 1 h before surgery until being killed 5 days later. Vehicle-treated recipients developed severe allograft failure with marked histologic damage in association with dense leucocyte infiltration (T cells, macrophages, neutrophils and NK cells) and deposition of IgM, IgG and C3. Immunostaining identified Syk expression by many infiltrating leucocytes. CC0482417 treatment significantly improved allograft function and reduced histologic damage, although allograft injury was still clearly evident. CC0482417 failed to prevent T-cell infiltration and activation within the allograft. However, CC0482417 significantly attenuated acute tubular necrosis, infiltration of macrophages and neutrophils and thrombosis of peritubular capillaries. In conclusion, this study identifies a role for Syk in acute renal allograft rejection. Syk inhibition may be a useful addition to T-cell-based immunotherapy in renal transplantation.

Keywords
humoral rejection, macrophage, neutrophil, Syk, T-cell activation

Major advances have been made in controlling T-cell-mediated allograft rejection (Nankivell & Alexander 2010). However, this success has revealed the presence of significant levels of humoral allograft rejection driven by pre-existing or *de novo* donor-specific antibodies which is poorly controlled by current therapies (Roberts *et al.* 2012). Thus, there is a major unmet medical need for new therapeutic options in the treatment of antibody-dependent allograft rejection.

Spleen tyrosine kinase (Syk) is an intracellular enzyme which is required for signal transduction via the B-cell receptor, Fc receptors, some leucocyte integrins, platelet GPVI and complement receptor 3 (Mocsai *et al.* 2010). Syk is widely expressed in leucocyte populations, except in mature T cells where the ZAP70 kinase is required for T-cell receptor signalling (Mocsai *et al.* 2010). Studies in mouse and rat models have demonstrated that small molecule inhibitors of Syk can suppress renal injury in models of native kidney disease, including lupus nephritis, nephrotoxic serum nephritis and autoimmune experimental glomerulonephritis (Bahjat *et al.* 2008; Smith *et al.* 2010; Ryan *et al.* 2011; McAdoo *et al.* 2014). These studies have shown the Syk blockade can inhibit neutrophil and platelet-mediated glomerular injury, macrophage-mediated renal injury and autoantibody production (Bahjat *et al.* 2008; Smith *et al.* 2010; Ryan *et al.* 2011; McAdoo *et al.* 2014). One study has examined Syk in allograft rejection in which piceatannol treatment (which blocks both Syk and ZAP70), together with cyclosporine A, was shown to provide long-term survival in a rat kidney allograft model (Fernandez *et al.*

2002). However, the role of Syk in allograft rejection has yet to be examined.

The aim of this study was to determine the contribution of Syk signalling in acute allograft rejection. This was investigated using a Syk inhibitor compound (CC0482417) in a rat model of acute renal allograft rejection.

Materials and methods

Syk inhibitor

The selective Syk inhibitor, CC0482417, was manufactured by Celgene (San Diego, CA, USA). In an enzyme assay, CC0482417 inhibits Syk with an IC50 of 3.1 nM. In a panel of 71 enzymes, the closest kinases inhibited were JAK2 (IC_{50} 15.9 nM), JAK1 (IC_{50} 16.5 nM) and JAK3 (IC_{50} 34.7 nM). CC0482417 has no activity against ZAP70. In cellular assays, CC0482417 inhibits anti-IgM-stimulated NFAT activation at an IC50 of 31 nM. The drug was prepared in 20% hydroxylpropyl-β-cyclodextrin vehicle (Sigma-Aldrich, Castle Hill, NSW, Australia) and administered by twice daily gavage at the maximum beneficial dose of 30 mg/kg.

Antibodies

Mouse anti-rat monoclonal antibodies used were the following: ED1 (CD68), RECA-1 (endothelium) (Serotec, Oxford, UK); RP1 (neutrophils) (Becton Dickinson, San Diego, CA, USA); and NKR-P1 (NK cells) (Cedarlane, Burlington, ON, Canada). Other mouse anti-rat antibodies were prepared in-house; OX-22 (B cells), R73 (rat αβ T-cell receptor), and NDS61 (CD25/IL-2Rα). Rabbit antibodies were against: fibrinogen (Santa Cruz Biotechnology, CA, USA) and Syk (Cell Signalling, Danvers, MA, USA). Biotinylated antibodies were goat anti-mouse IgG (Zymed, San Francisco, CA, USA) and goat anti-rabbit IgG (Invitrogen, CA, USA), which were detected using a Vectastain ABC kit (Vector Laboratories, CA, USA). FITC (Fluorescein isothiocyanate)-conjugated goat polyclonal antibodies were against rat IgG (Sigma-Aldrich), rat IgM (Bethyl, Montgomery, TX, USA) and rat C3 (Cappel, Malvern, PA, USA).

Rat model of acute renal allograft rejection

Orthotopic transplantation of a Wistar kidney into bilateral nephrectomized Sprague Dawley (SD) recipients was performed as previously described (Kerr *et al.* 1994; Ma *et al.* 2013). Briefly, the donor Wistar kidney was isolated and perfused with ice-cold Ross solution and then implanted orthotopically in recipient Sprague–Dawley rats with end-to-end anastomosis of renal artery (sleeve), vein (stent) and ureter (stent). Groups of seven allograft recipient rats were given the Syk inhibitor or vehicle alone 1 h before surgery and then by twice daily oral gavage until killed at day 5 after surgery. Normal Sprague–Dawley rats were used as controls. Animals were obtained from the Monash Animal Research Platform, Australia, and the experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee. Serum creatinine was analysed by the Department of Biochemistry at Monash Medical Centre.

Histologic analysis of allograft rejection

Paraffin sections of formalin-fixed tissue were stained with periodic acid-Schiff and haematoxylin. Slides were coded and assessed in a blinded manner. Injured tubules were identified with one or more of the following pathological changes: tubular atrophy, loss of brush border, loss of nuclei, basement membrane disruption and detachment of epithelial cells. Over 600 tubules were examined in each animal using 12 high power fields (×400) in the renal cortex and results expressed as percentage of injured tubules.

Immunohistochemistry

Immunoperoxidase staining with ED1, Syk and fibrinogen antibodies was performed on formalin-fixed paraffin sections using antigen retrieval followed by a three-layer avidin biotin peroxidase complex (ABC) staining method (Lan *et al.* 1995). Immunoperoxidase staining with R73, RECA-1, OX22, RP1, NKR-P1 and NDS61 antibodies was performed on cryostat sections of tissue fixed in 2% paraformaldehyde-lysine-periodate using the ABC method.

Quantification of immunohistochemistry

Allograft infiltration of RP1 + neutrophils, OX22 + B cells and NKR-P1 + NK cells was assessed in glomeruli and in the interstitial area of the entire cortex of allografts under medium power (×250) and expressed as the number of positive cells per mm^2. The peritubular capillary rarefaction index of peritubular capillary sparseness (0–100%) was determined by counting the numbers of squares in 12 × 16 grids containing RECA-1+ peritubular capillaries in at least 12 consecutive medium-power (×160) fields. The same method was employed to quantify the number of grid squares containing fibrinogen staining in peritubular capillaries. All scoring was performed on coded slides.

Immunofluorescence

Immunofluorescence staining was performed on sections of snap-frozen tissue using FITC-conjugated antibodies to rat IgG, IgM and C3. Slides were photographed and quantified by image analysis using Image-Pro software (Media Cybernetics, Rockville, MD, USA).

Real-time RT-PCR

Kidney and spleen samples were snap-frozen and stored at –80°C until RNA was extracted using the RiboPure RNA isolation kit (Ambion, Austin, TX, USA). cDNA was prepared from total RNA by reverse transcription using random hexamer primers and Superscript II (Invitrogen,

Carlsbad, CA, USA). Real-time RT-PCR was performed on a StepOne machine (Applied Biosystems, Mulgrave, VIC, Australia) using primers plus Taqman probes with thermal cycling conditions of 37°C for 10 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 20 s. The primers and probes for detection of KIM-1, CD3, granzyme B, IFN-γ, FoxP3, CD68, NOS2, MMP-12, TNF-α, IL-12b, arginase-1, CD163, CD206, IL-10 and MCP-1 have been described previously (Ma *et al.* 2009, 2013; Han *et al.* 2013), except for the IL-2 primers and probe which were purchased from Applied Biosystems. The relative amount of mRNA was calculated using the comparative Ct method. All amplicons were normalized against the 18S RNA internal control (Applied Biosystems).

Statistics

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

Results

CC0482417 reduced the severity of acute renal allograft rejection

Bilateral nephrectomised Sprague–Dawley rats received an orthotopic Wistar rat kidney allograft. Vehicle-treated recipients developed severe renal allograft rejection on day 5 as shown by a 10-fold increase in serum creatinine levels (Figure 1a). Allograft histology revealed widespread acute tubular injury, diffuse heavy leucocytic infiltration with perivascular accentuation, peritubular capillaritis and glomerulopathy (Figure 1d). Most tubules exhibited histologic damage, while the marked up-regulation of KIM-1 was consistent with severe tubular damage (Figure 1b and c). Immunohistochemistry staining confirmed Syk expression in many infiltrating leucocytes in vehicle-treated allografts (Figure 1f).

Treatment with CC0482417 reduced renal allograft rejection as shown by the significant reduction in serum creatinine levels, although this was still fivefold elevated above normal (Figure 1a). Histology also showed a significant reduction in allograft damage, although moderate leucocyte infiltration was still evident (Figure 1b).

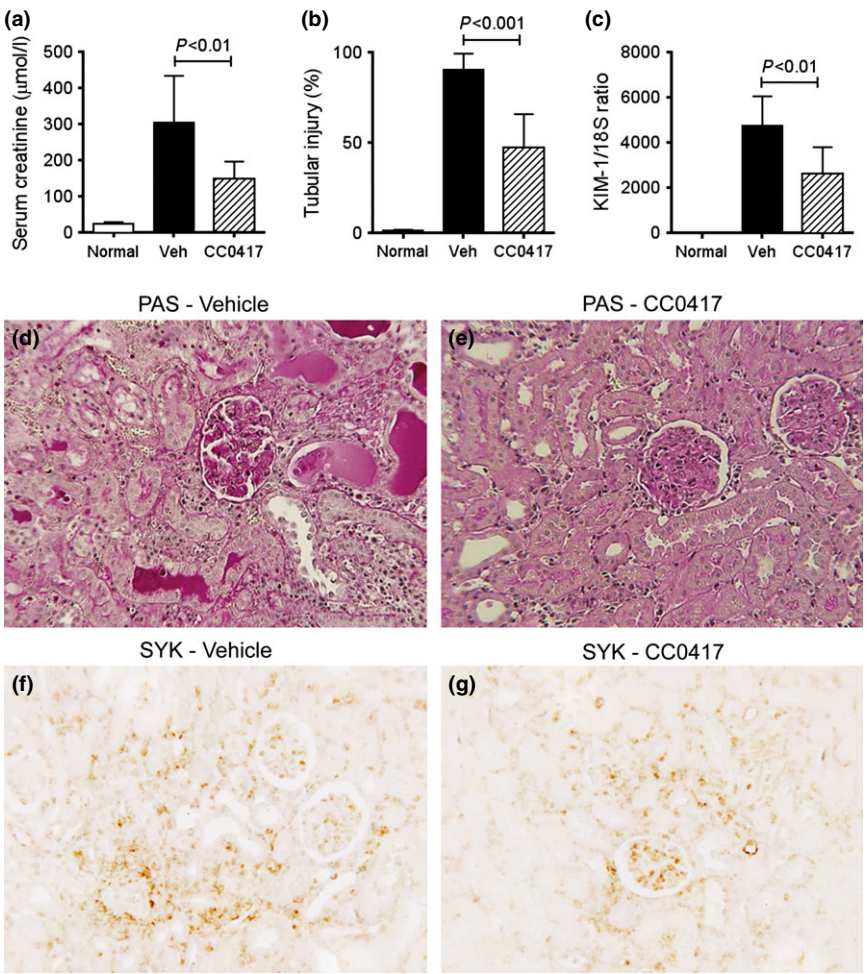

Figure 1 CC0482417 (CC0417) reduced renal allograft damage. Graphs show (a) serum creatinine; (b) tubular injury; (c) KIM-1 mRNA levels. PAS staining of (d) vehicle- and (e) CC4017-treated allografts. Immunostaining for Syk in (f) vehicle- and (g) CC0417-treated allografts. Original magnification, ×250 (d and e) and ×160 (f and g).

Quantitative analysis showed a partial, but significant, reduction in tubular damage on the basis of histology and KIM-1 mRNA levels (Figure 1b and c). In addition, numerous Syk-expressing leucocytes were still evident in CC0482417-treated allografts (Figure 1 g).

CC0482417 did not affect allograft T-cell infiltration or activation

A prominent T-cell infiltrate was seen in vehicle-treated allografts on the basis of immunohistochemistry staining of R73 + T cells and PCR analysis of CD3 mRNA levels (Figure 2a and c). Many IL-2Rα+ (CD25+) T cells were evident in vehicle-treated allografts which are predominantly activated effector T cells (Figure 2d). T-cell activation within these allografts was also shown by the prominent up-regulation of mRNA levels for IL-2 (a general marker of T-cell activation), granzyme B (a marker of activated cytotoxic T cells and natural killer cells) and IFN-γ (a marker of activated T_h1-type T cells), while an increase in FoxP3 mRNA indicated infiltration of regulatory T cells (Figure 2f–i). CC0482417 treatment had no effect upon T-cell infiltration, parameters of T-cell activation or on the infiltration of regulatory T cells (Figure 2).

CC0482417 reduced macrophage infiltration in acute allograft rejection

Immunohistochemistry staining identified an enormous macrophage infiltrate throughout vehicle-treated allografts (glomerular, interstitial and perivascular) which was also evident by the prominent up-regulation of CD68 mRNA levels (Figure 3a and c). PCR analysis of vehicle-treated renal allografts showed marked up-regulation of markers of both classically activated M1-type macrophages (NOS2, MMP-12, TNF-α and IL-12) and alternatively activated M2-type macrophages (Arg-1, CD163, CD206, IL-10) (Figure 3d–k). CC0482417 treatment reduced the overall macrophage infiltrate by approximately 40% (Figure 3b and c). The reduced expression of most markers of M1- and M2-type macrophage activation seen with CC0482417 treatment was proportional to the reduction in the total macrophage infiltrate, except for the M2 marker, IL-10, whose expression was increased twofold ($P < 0.05$) relative to CD68 mRNA levels in drug-treated allografts (Figure 3d–k). In addition, CC0482417 reduced MCP-1 mRNA levels which may have contributed to the reduction in overall macrophage infiltration (Figure 3l).

CC0482417 did not prevent antibody deposition in renal allografts

Humoral rejection involves the deposition of donor-specific antibodies within the allograft. Confocal microscopy identified prominent rat IgM and IgG deposition along the glomerular endothelium, peritubular capillaries and arterioles which was absent in normal rats (Figure 4a,b,d,e). CC0482417 treatment did not affect the deposition of rat IgM or IgG within allografts (Figure 4c and f–h). In addition, CC0482417 treatment did not affect the glomerular deposition of C3 which was evident in allografts (Figure 4i).

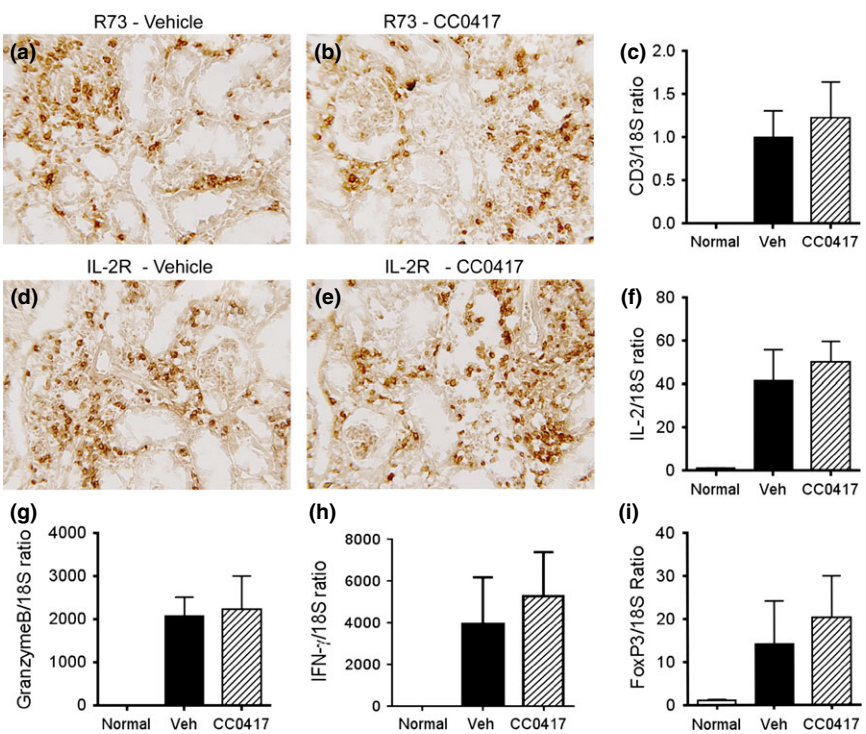

Figure 2 CC0482417 (CC0417) has no effect upon T-cell infiltration or activation. Immunostaining for R73 + T cells in (a) vehicle- and (b) CC4017-treated allografts. (c) CD3 mRNA levels. Immunostaining for the IL-2Rα (CD25) in (d) vehicle- and (e) CC0417-treated allografts. PCR analysis of mRNA levels for: (f) IL-2, (g) granzyme B, (h) IFN-γ and (i) FoxP3. Original magnification, ×250 (a, b, d, e).

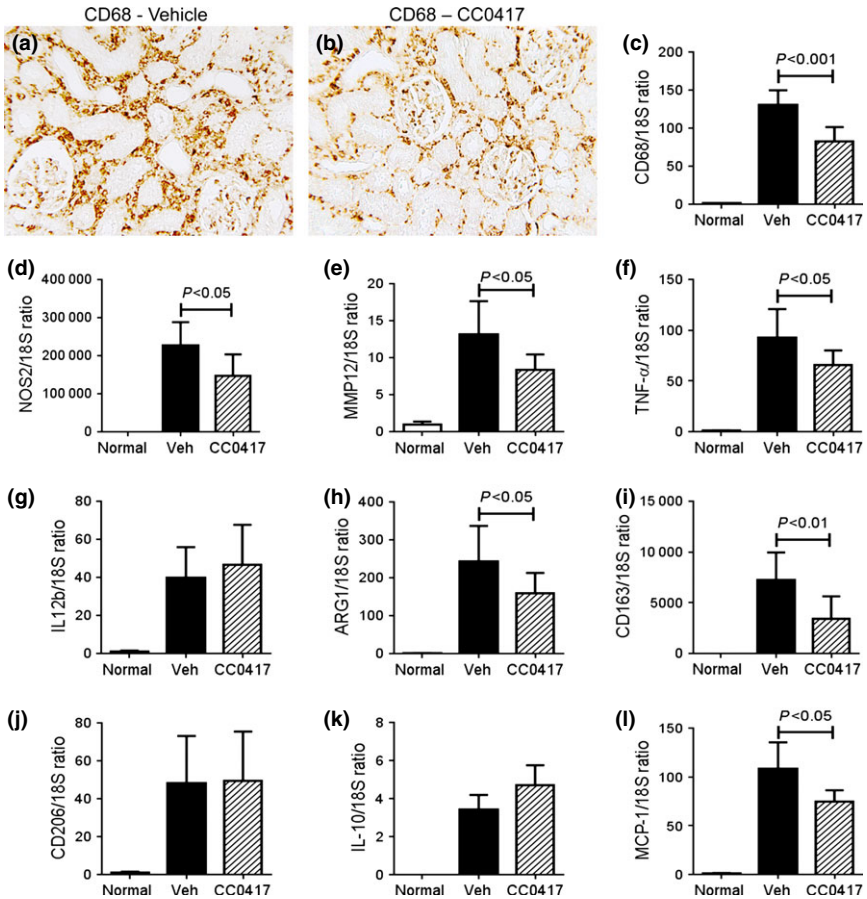

Figure 3 CC0482417 (CC0417) reduced macrophage infiltration in renal allografts. Immunostaining for ED1 (CD68+) macrophages in (a) vehicle- and (b) CC0417-treated allografts. PCR analysis of mRNA levels of: (c) CD68, (d) NOS2, (e) MMP-12, (f) TNF-α, (g) IL-12b, (h) Arginase-1, (i) CD163, (j) CD206, (k) IL-10 and (l) MCP-1. Original magnification, ×250 (a, b).

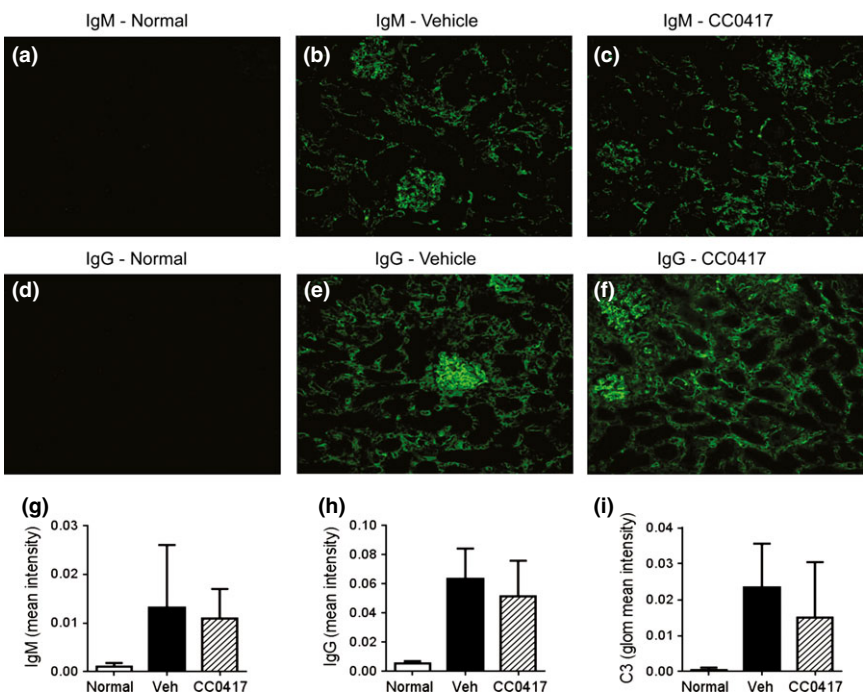

Figure 4 Effect of CC0482417 (CC0417) on antibody and complement deposition in renal allografts. Confocal microscopy shows deposition of rat IgM in: (a) normal rat kidney and in (b) vehicle- and (c) CC0417-treated allografts. Deposition of rat IgG is shown in: (d) normal rat kidney and in (e) vehicle- and (f) CC0417-treated allografts. Graphs show quantification of the fluorescence signal for deposition of: (g) rat IgM, (h) rat IgG and (i) rat C3. Original magnification, ×250 (c–h).

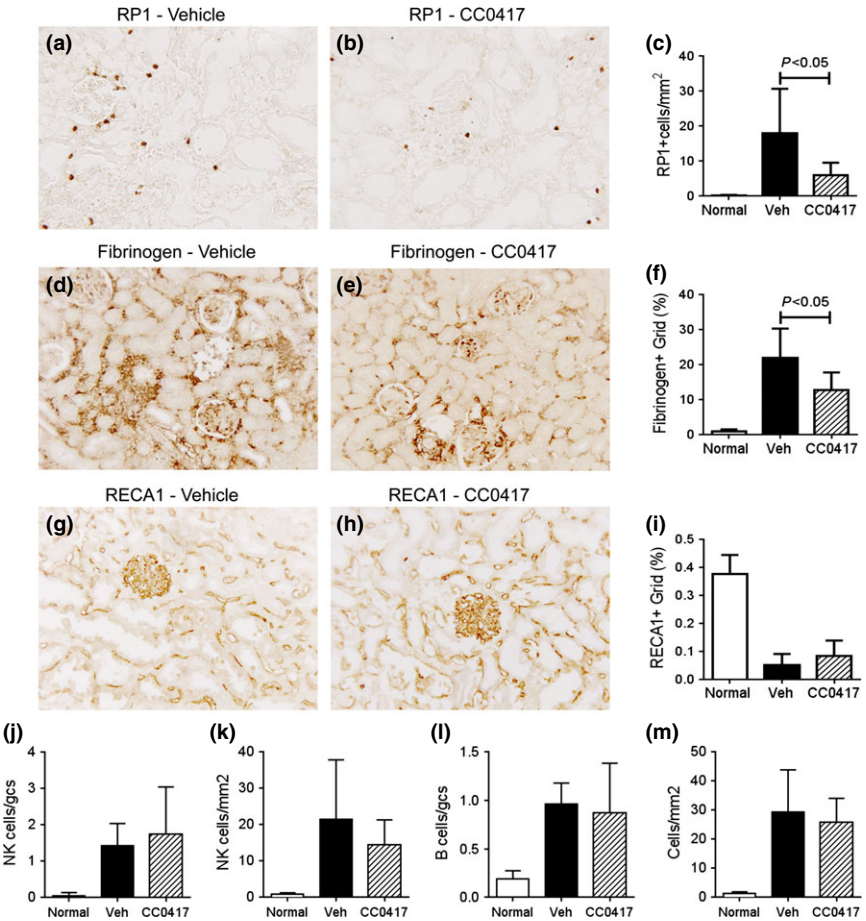

Figure 5 Effect of CC0482417 (CC0417) on parameters of humoral allograft rejection. Immunostaining for RP1 + neutrophils in: (a) vehicle- and (b) CC0417-treated allografts. (c) Quantification of neutrophil infiltration. Immunostaining for fibrinogen deposition in: (d) vehicle- and (e) CC0417-treated allografts. (f) Quantification of the percentage of grid squares containing fibrinogen+ peritubular. Immunostaining for RECA-1 + endothelial cells in: (g) vehicle- and (h) CC0417-treated allografts. (i) Quantification of the percentage of grid squares containing RECA-1 + peritubular capillaries. Quantification of: (j) glomerular NK cells, (k) interstitial NK cells, (l) glomerular B cells and (m) interstitial B cells. Original magnification, ×160 (a, b, d, e, g, h).

Effect of CC0482417 on other features of allograft rejection

We examined a number of other features which are associated with antibody-mediated rejection. CC0482417 significantly reduced neutrophil infiltration in renal allografts (Figure 5a–c). However, the NK-cell and B-cell infiltration evident in vehicle-treated allografts was not affected by CC0482417 (Figure 5j–m). Substantial thrombosis was evident in vehicle-treated allografts, being most prominent in peritubular capillaries as assessed by fibrinogen immunostaining (Figure 5d). This was significantly reduced by CC0482417 treatment (Figure 5e and f). In addition, there was a significant loss of peritubular capillaries in vehicle-treated allografts compared to normal rat kidney; however, CC0482417 treatment did not provide significant protection against this loss (Figure 5g–i).

Discussion

This study has identified a pathological role for Syk in acute renal allograft rejection. Syk inhibition had no effect upon the T-cell allograft response, but inhibited some features associated with antibody-mediated rejection (acute tubular damage, macrophage and neutrophil infiltration and thrombosis).

Most studies of acute allograft rejection focus on the role of T cells in allograft destruction. However, a significant humoral immune response is also involved in this process as shown in the current study by prominent IgM and IgG deposition along the allograft endothelium together with complement deposition. Syk is required for B-cell receptor signalling (Mocsai *et al.* 2010). However, few studies have examined the role of Syk in antibody responses, largely due the failure of B-cell development in mice lacking the Syk gene in the bone marrow compartment (Cheng *et al.* 1995; Turner *et al.* 1995). One recent study showed that treatment with a Syk inhibitor (fostamatinib) was effective in preventing autoantibody production in response to immunisation with the Goodpasture antigen in complete Freund's adjuvant (McAdoo *et al.* 2014). In the current study, CC0482417 treatment failed to inhibit antibody deposition in the allograft. Whether this is due to insufficient drug levels to inhibit the very high Syk levels present in B cells is not clear. However, we can conclude that the protective effect of CC0482417 in the model of allograft rejection is not simply due to reduced antibody deposition and complement activation. Rather, CC0482417 treatment appears to have inhibited some of the downstream effects following the deposition of humoral reactants within the allograft.

Macrophage infiltration is a prominent feature of the acute phase of renal allograft rejection in non-immunosuppressed animals (Kerr *et al.* 1994). These infiltrating cells can be activated via several mechanisms, including Fc and complement receptors, T-cell- and NK-cell-derived cytokines such as IFN-γ, and release of endogenous ligands for danger-associated molecular pattern (DAMP) receptors (Chadban *et al.* 2010). We found that CC0482417 treatment significantly reduced the degree of macrophage infiltration, but had relatively little impact upon the M1/M2 phenotype of the infiltrating macrophages. The reduction in macrophage infiltration may be due to CC0482417 inhibiting Fc receptor-based recruitment, as well as the partial reduction in allograft MCP-1 production. The lack of effect of CC0482417 treatment on the pro-inflammatory M1-type macrophage response may be due to the unaltered production of IFN-γ by infiltrating T cells and NK cells. Indeed, our recent studies in a similar model of rat renal allograft rejection identified a role for macrophages in cellular but not humoral-mediated rejection (Ma *et al.* 2013).

Neutrophils are considered to be important effectors of antibody-mediated allograft injury (Murata & Baldwin 2009). Syk is involved in the recruitment and activation of neutrophils via Fcγ receptors (Florey *et al.* 2007; Tsuboi *et al.* 2008). Thus, the reduced allograft damage seen with CC0482417 treatment may be, in part, due to the reduced neutrophil infiltration and/or activation within the allograft. NK cells can also induce allograft injury via direct mechanisms (antibody-dependent cell-mediated cytotoxicity) and indirect mechanisms (cytokine production to activate other leucocytes) (Rocha *et al.* 2003; Murata & Baldwin 2009). Although CC0482417 did not affect allograft infiltration of NK cells or affect up-regulation of IFN-γ expression, it is possible that the treatment suppressed NK-cell-mediated allograft injury via antibody-dependent cell-mediated cytotoxicity. Finally, thrombosis is an important mechanism of allograft injury (Meehan *et al.* 2003; Murata & Baldwin 2009). Syk is required for platelet activation and thrombosis via glycoprotein VI-FcγR-mediated signalling (Kim *et al.* 2013; Ozaki *et al.* 2013), and Syk inhibitor treatment has been shown to suppress thrombosis and renal injury in antibody-dependent native kidney disease (Ryan *et al.* 2011). Thus, inhibition of platelet activation may also have contributed to the reduction in capillary thrombosis seen with CC0482417 treatment of renal allografts.

An alternative mechanism by which Syk activation may have contributed to allograft rejection is via Toll-like receptor (TLR) signalling in the innate immune response. Syk is thought to be directly involved in TLR4 signalling (Miller *et al.* 2012). This is potentially important as TLR4 signalling promotes renal ischaemia/reperfusion injury (Wu *et al.* 2007), and this type of injury is unavoidable in kidney transplantation. In addition, it has been shown that TLR2/4 expression in the mouse renal allograft contributes to both macrophage infiltration and chronic allograft rejection (Wang *et al.* 2010), thus providing a potential mechanism by which CC0482417 may have reduced acute renal allograft injury in the present study.

One important finding in the current study was the lack of effect of CC0482417 on the allograft T-cell response. Using both immunostaining and PCR analysis, we identified prominent T-cell infiltration and activation within the allograft which was unaffected by CC0482417 treatment. This is consistent with the first genetic studies of Syk in which reconstitution of immunodeficient mice with Syk-deficient foetal liver cells did not affect the development of normal mature CD4 and CD8 T-cell populations (Cheng *et al.* 1995; Turner *et al.* 1995), and that human mature T cells also lack Syk expression (Feldman *et al.* 2008). However, a role for Syk in early thymocyte development has been described, although this does not prevent the development of mature T cells (Chan *et al.* 1994; Palacios & Weiss 2007), and the abnormal T cells present in systemic lupus erythematosus and in T cell lymphomas can utilise Syk signalling (Feldman *et al.* 2008; Grammatikos *et al.* 2013). One issue that complicates the understanding of Syk function is that the Syk inhibitor drug used in most animal studies, fostamatinib, is non-selective and inhibits 14 other kinases more potently than Syk, including Jak2 and ZAP70 which play critical roles in T-cell activation (Davis *et al.* 2011; Currie *et al.* 2014; Thoma *et al.* 2014). Indeed, the active metabolite of fostamatinib, R406, was originally described as having activity against IL-2-induced T-cell proliferation and IL-4-induced B-cell activation, indicating blockade of JAK/STAT signalling (Braselmann *et al.* 2006). A recent study showed that fostamatinib can suppress a mouse model of bone marrow transplant chronic graft-versus-host disease; however, this protection was attributed to blockade of the proliferation and activation of effector CD4+ T cells (Le Huu *et al.* 2014). Thus, caution is needed when interpreting studies of fostamatinib treatment in disease models.

In the current study, immunostaining confirmed Syk expression by infiltrating leucocytes. We have previously identified Syk activation in infiltrating leucocytes in rat models of kidney disease and kidney transplantation based on phosphorylation of the activation loop of the Syk enzyme (Ryan *et al.* 2011; Ma *et al.* 2013); however, recent batches of this anti-human p-Syk polyclonal antibody have been unable to detect Syk activation in rat tissue sections, and thus we were unable to confirm that CC0482417 inhibited Syk activation in the rat allograft model. Further studies to investigate the role of Syk in humoral rejection are needed. The protective effect of Syk blockade needs to be investigated in a model of antibody-mediated rejection in the absence of T-cell-mediated graft damage. In addition, the potential for combined complement and Syk blockade in antibody-mediated rejection should be examined. Finally, the use of conditional Syk gene deletion is required to both confirm the role of Syk in allograft rejection and identify the specific cell types in which Syk activation is critical.

Finally, one limitation of the study is that the potential for Syk blockade to provide a survival benefit was not investigated. All animals were killed on day 5 post-trans-

plant to avoid the anticipated death of vehicle-treated rats due to allograft failure on day 7. However, it is unlikely that Syk blockade would have prolonged allograft survival in this model by more than a few days at most as allograft dysfunction and prominent T cell-infiltration and activation were already evident in drug-treated recipient rats on day 5.

In conclusion, this study has identified a role for Syk in acute renal allograft rejection which is independent of T-cell activation. Thus, Syk inhibition may be a useful addition to T-cell-based immunotherapy in renal transplantation.

Acknowledgements

The authors wish to thank Dr Brian Howden for technical assistance with rat transplantation.

Ethical Approval statement

All animal experiments were approved by the Monash Medical Centre Animal Ethics Committee and performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes.

Funding source

This study was funded by a National Health and Medical Research Council of Australia Project Grant (DN-P, FM) and a National Health and Medical Research Council of Australia Senior Research Fellowship (DN-P).

Conflict of interest

KB is an employee of Celgene who supplied the CC0417 compound used in this study.

References

Bahjat F.R., Pine P.R., Reitsma A. *et al.* (2008) An orally bioavailable spleen tyrosine kinase inhibitor delays disease progression and prolongs survival in murine lupus. *Arthritis Rheum.* **58**, 1433–1444.

Braselmann S., Taylor V., Zhao H. *et al.* (2006) R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-mediated inflammation. *J. Pharmacol. Exp. Ther.* **319**, 998–1008.

Chadban S.J., Wu H. & Hughes J. (2010) Macrophages and kidney transplantation. *Semin. Nephrol.* **30**, 278–289.

Chan A.C., van Oers N.S., Tran A. *et al.* (1994) Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *J. Immunol.* **152**, 4758–4766.

Cheng A.M., Rowley B., Pao W., Hayday A., Bolen J.B. & Pawson T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* **378**, 303–306.

Currie K.S., Kropf J.E., Lee T. *et al.* (2014) Discovery of GS-9973, a selective and orally efficacious inhibitor of spleen tyrosine kinase. *J. Med. Chem.* **57**, 3856–3873.

Davis M.I., Hunt J.P., Herrgard S. *et al.* (2011) Comprehensive analysis of kinase inhibitor selectivity. *Nature Biotechnol.* **29**, 1046–1051.

Feldman A.L., Sun D.X., Law M.E. *et al.* (2008) Overexpression of Syk tyrosine kinase in peripheral T-cell lymphomas. *Leukemia* **22**, 1139–1143.

Fernandez L.A., Torrealba J., Yagci G. *et al.* (2002) Piceatannol in combination with low doses of cyclosporine A prolongs kidney allograft survival in a stringent rat transplantation model. *Transplantation* **74**, 1609–1617.

Florey O.J., Johns M., Esho O.O., Mason J.C. & Haskard D.O. (2007) Antiendothelial cell antibodies mediate enhanced leukocyte adhesion to cytokine-activated endothelial cells through a novel mechanism requiring cooperation between Fc{gamma}RIIa and CXCR1/2. *Blood* **109**, 3881–3889.

Grammatikos A.P., Ghosh D., Devlin A., Kyttaris V.C. & Tsokos G.C. (2013) Spleen tyrosine kinase (Syk) regulates systemic lupus erythematosus (SLE) T cell signaling. *PLoS One* **8**, e74550.

Han Y., Ma F.Y., Tesch G.H., Manthey C.L. & Nikolic-Paterson D.J. (2013) Role of macrophages in the fibrotic phase of rat crescentic glomerulonephritis. *Am. J. Physiol. Renal. Physiol.* **304**, F1043–F1053.

Kerr P.G., Nikolic-Paterson D.J., Lan H.Y., Tesch G., Rainone S. & Atkins R.C. (1994) Deoxyspergualin suppresses local macrophage proliferation in rat renal allograft rejection. *Transplantation* **58**, 596–601.

Kim S., Dangelmaier C., Bhavanasi D. *et al.* (2013) RhoG protein regulates glycoprotein VI-Fc receptor gamma-chain complex-mediated platelet activation and thrombus formation. *J. Biol. Chem.* **288**, 34230–34238.

Lan H.Y., Mu W., Nikolic-Paterson D.J. & Atkins R.C. (1995) A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J. Histochem. Cytochem.* **43**, 97–102.

Le Huu D., Kimura H., Date M. *et al.* (2014) Blockade of Syk ameliorates the development of murine sclerodermatous chronic graft-versus-host disease. *J. Dermatol. Sci.* **74**, 214–221.

Ma F.Y., Flanc R.S., Tesch G.H., Bennett B.L., Friedman G.C. & Nikolic-Paterson D.J. (2009) Blockade of the c-Jun amino terminal kinase prevents crescent formation and halts established anti-GBM glomerulonephritis in the rat. *Lab. Invest.* **89**, 470–484.

Ma F.Y., Woodman N., Mulley W.R., Kanellis J. & Nikolic-Paterson D.J. (2013) Macrophages contribute to cellular but not humoral mechanisms of acute rejection in rat renal allografts. *Transplantation* **96**, 949–957.

McAdoo S.P., Reynolds J., Bhangal G. *et al.* (2014) Spleen tyrosine kinase inhibition attenuates autoantibody production and reverses experimental autoimmune GN. *J. Am. Soc. Nephrol.* **25**, 2291–2302.

Meehan S.M., Limsrichamrern S., Manaligod J.R. *et al.* (2003) Platelets and capillary injury in acute humoral rejection of renal allografts. *Human Pathol.* **34**, 533–540.

Miller Y.I., Choi S.H., Wiesner P. & Bae Y.S. (2012) The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL. *Br. J. Pharmacol.* **167**, 990–999.

Mocsai A., Ruland J. & Tybulewicz V.L. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10**, 387–402.

Murata K. & Baldwin W.M. 3rd (2009) Mechanisms of complement activation, C4d deposition, and their contribution to the

pathogenesis of antibody-mediated rejection. *Transplant Rev.* **23**, 139–150.

Nankivell B.J. & Alexander S.I. (2010) Rejection of the kidney allograft. *N. Engl. J. Med.* **363**, 1451–1462.

Ozaki Y., Suzuki-Inoue K. & Inoue O. (2013) Platelet receptors activated via mulitmerization: glycoprotein VI, GPIb-IX-V, and CLEC-2. *J. Thromb. Haemost.* **11**(Suppl 1), 330–339.

Palacios E.H. & Weiss A. (2007) Distinct roles for Syk and ZAP-70 during early thymocyte development. *J. Exp. Med.* **204**, 1703–1715.

Roberts D.M., Jiang S.H. & Chadban S.J. (2012) The treatment of acute antibody-mediated rejection in kidney transplant recipients-a systematic review. *Transplantation* **94**, 775–783.

Rocha P.N., Plumb T.J., Crowley S.D. & Coffman T.M. (2003) Effector mechanisms in transplant rejection. *Immunol. Rev.* **196**, 51–64.

Ryan J., Ma F.Y., Kanellis J., Delgado M., Blease K. & Nikolic-Paterson D.J. (2011) Spleen tyrosine kinase promotes acute neutrophil-mediated glomerular injury via activation of JNK and p38 MAPK in rat nephrotoxic serum nephritis. *Lab. Invest.* **91**, 1727–1738.

Smith J., McDaid J.P., Bhangal G. *et al.* (2010) A spleen tyrosine kinase inhibitor reduces the severity of established glomerulonephritis. *J. Am. Soc. Nephrol.* **21**, 231–236.

Thoma G., Blanz J., Buhlmayer P. *et al.* (2014) Syk inhibitors with high potency in presence of blood. *Bioorg. Med. Chem. Lett.* **24**, 2278–2282.

Tsuboi N., Asano K., Lauterbach M. & Mayadas T.N. (2008) Human neutrophil Fcgamma receptors initiate and play specialized nonredundant roles in antibody-mediated inflammatory diseases. *Immunity* **28**, 833–846.

Turner M., Mee P.J., Costello P.S. *et al.* (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* **378**, 298–302.

Wang S., Schmaderer C., Kiss E. *et al.* (2010) Recipient Toll-like receptors contribute to chronic graft dysfunction by both MyD88- and TRIF-dependent signaling. *Dis. Model. Mech.* **3**, 92–103.

Wu H., Chen G., Wyburn K.R. *et al.* (2007) TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest.* **117**, 2847–2859.