SYK inhibition attenuates autoantibody production and reverses Experimental Autoimmune Glomerulonephritis

SUPPLEMENTARY INFORMATION

Includes: Supplementary Figures

Detailed Methods
Supplementary Figures

Supplementary Figure 1

Complete histology counts for (A) the preventative *in vivo* study, and (B) the established disease *in vivo* study, showing the distribution of severely abnormal (>50% of the glomerular tuft affected by necrosis or crescent formation), abnormal (any abnormality of the remaining glomeruli) and normal glomeruli across all groups.
Detailed Methods

Study Approval

Human renal tissue samples were obtained from patients under local ethics committee approval (04/Q0406/25 Hammersmith and Queen Charlotte’s & Chelsea Hospitals Research Ethics Committee). Lymph node samples were provided the Imperial College Healthcare NHS Trust Tissue Bank. Animal studies were carried out with local ethics committee approval (Central Biomedical Services, Imperial College London) and in accordance with the UK Animals (Scientific Procedures Act) 1986.

Immunohistochemistry for SYK

Immunohistochemistry (IHC) on human tissue was performed on renal biopsy sections, surplus to clinical need, from patients with anti-GBM disease and thin basement membrane disease, as identified using a central renal pathology database at Imperial College Renal and Transplant Centre, Hammersmith Hospital, London UK. IHC on rat tissue was performed on specimens taken from animals 18 days after induction of EAG. For double staining, sections taken from animals 28 days after disease induction were used.

All IHC was performed on formalin-fixed paraffin-embedded tissues. Sections were subject to heat-induced antigen retrieval in 0.1M citrate buffer (pH 6) and then sequentially blocked in 0.3% hydrogen peroxide and 20% goat serum (Dako Ltd, Cambridgeshire UK). Sections were then incubated with the following primary antibodies overnight at 4°C: total SYK N-19 (Santa Cruz Biotechnology, Dallas TX; dilution 1:500); human phosphorylated SYK Tyr
525/526 (Cell Signaling Technology, Danvers MA; dilution 1:20); rat phosphorylated SYK Try323 (Abcam, Cambridge UK; dilution 1:50). After washing, sections were incubated in a secondary polymer-HRP system (EnVision +; Dako) and developed in 3,3-diaminobenzidine (DAB), according to the manufacturer’s instructions. The sections were then rinsed, counterstained with filtered in Harris’ haematoxylin (CellPath, Powys UK), dehydrated and mounted with DPX mounting medium (Fisher Scientific, Leicester UK).

For double staining, sections were first stained for phosphorylated SYK as described above. Following development in DAB, the sections were sequentially rinsed, re-subjected to heat-induced antigen retrieval (0.1M citrate buffer, pH 6.0), blocked with 20% goat serum (Dako), incubated with mouse anti-rat ED1 primary antibody (Serotec, Oxford UK; dilution 1:500) at room temperature for 1 hour, washed, then incubated with a biotinylated goat anti-mouse immunoglobulin secondary antibody (SigmaAldrich, St Louis MO; dilution 1:100) for 1 hour, then ALP-conjugated streptavidin (Roche Diagnostics, Mannheim Germany; dilution 1:100) for 30 minutes, before final development using the Vector Blue ALP Substrate Kit III (Vector Labs, Burlingame CA) according to manufacturer’s specifications. Slides were mounted in AquaPerm (Thermo Scientific, Pittsburg PA) without counterstaining, and coverslips placed.

**SYK inhibitors**

The SYK inhibitor R406, and its orally bioavailable prodrug R788 (fostamatinib disodium) were provided by Rigel Pharmaceuticals (South San Francisco, CA) and AstraZeneca (London UK). The details of these molecules have been reported previously.1, 2
For *in vivo* experiments, fostamatinib was reconstituted in vehicle formulation (0.1% carboxymethylcellulose, 0.1% methylparaben sodium, 0.02% propylparaben sodium, in distilled water) to a concentration of 8mg/ml. Treated animals received 40mg/kg body weight, administered by twice daily oral gavage. Control animals received an equivalent volume and schedule of vehicle formulation. This dose was selected based on our previous dose-response studies showing biological efficacy without toxicity in NTN, and consistent with doses used in other *in vivo* studies in murine lupus.

For *in vitro* experiments, R406 was reconstituted in 0.1% dimethyl sulphoxide (DMSO; Sigma) in cell culture media as detailed below.

**Production of α3(IV)NC1**

Recombinant rat α3(IV)NC1 was produced as previously reported. Briefly, α3(IV)NC1 was produced from a HEK293 cell line stably transfected with cDNA encoding the full-length rat α3(IV)NC1. Secreted protein was purified from cell culture supernatant by affinity chromatography using an anti-FLAG M2 affinity column (Sigma) and characterized by Western blotting.

**Experimental Autoimmune Glomerulonephritis (EAG)**

Animals were purchased from Charles River (Margate UK) and housed in standard conditions with free access to normal laboratory diet and water. Female Wistar Kyoto (WKY) rats aged 6 weeks and with approximate body weight 100-120g were used for all experiments. For inhibitor studies, n=8 animals per group. To obtain comparative histology
in treated and untreated animals at day 18, and additional two groups (n=4) were used. One animal in the preventative *in vivo* study was sacrificed early due to an immediate complication of oral gavage; results for 7 animals in the fostamatinib group are therefore reported.

Disease was induced by immunizing rats with 100mcg recombinant rat α3(IV)NC1 in an equal volume of complete Freund’s adjuvant (CFA; Sigma), administered by intramuscular injection to each flank.

Urine was collected by housing rats in metabolic cages for 24 hours with free access to food and water, on days 9, 18, 27 and 36. Serum was collected by superficial tail puncture under isofluorane general anaesthesia on days 9, 18 and 27. At the termination of each experiment, rats were killed by terminal bleed under isofluorane general anaesthesia, and serum and tissue collected for analysis.

**Assessment of Lung Injury**

Lungs were scored by visual inspection at the time of cull to assess severity of pulmonary haemorrhage, using the following scale: 0 points – normal macroscopic lung appearances; 1 point – fewer than 10 visible petechiae; 2 points – greater than 10 visible petechiae; 4 points – large areas of infarct of haemorrhage.

Lung tissue was then collected for histological analysis by fixation in neutral buffered 10% formalin overnight, and transferred to 70% ethanol and processed to paraffin blocks. 4um sections were stained with Perls’ Prussian blue, without counterstain, to enable identification
of haemosiderin-laden cells. These were quantified by a blinded observer using automated image analysis software (ImagePro Plus, Media Cybernetics, Bethesda MD) to measure the proportion of Perls’ stained cells across 5 random high-power fields of lung sections from each animal, and expressed as the mean proportion per animal.

**Assessment of Renal Injury**

Urinary protein was quantified using sulphosalicylic acid method, as described previously.\textsuperscript{5} Haematuria was quantified by dipstick analysis (Multistix 8 SG, Siemens Healthcare Diagnostics, Rarrytown NY). Serum urea was kindly measured by Dr Olatunji Rowland in the Department of Clinical Biochemistry, Hammersmith Hospital, London UK, using an Olympus AU700 analyser (Olympus, Eastleigh UK). Kidney tissue was collected for histological analysis at time of cull by fixation in neutral buffered 10% formalin overnight, then transferred to 70% ethanol and processed to paraffin blocks. 4um sections were stained with periodic acid Schiff (PAS) reagent, haematoxylin and eosin (H&E) and Jones methenamine silver stain for assessment of glomerular injury. The severity of disease was graded in 50 consecutive glomeruli examined by light microscopy, by a blinded observer, as severe (>50% tuft affected by necrosis or crescent formation), abnormal (<50% tuft affected by necrosis or crescent; any degree of hypercellularity), or normal. Results are expressed as the proportion of severely abnormal glomeruli per animal.

**Immunohistochemistry of EAG tissue**

Immunostaining for ED-1 and CD8 positive cells was performed on formalin-fixed paraffin embedded kidney tissue. Sections were subject to heat-induced antigen retrieval in 0.1M
citrate buffer (pH 6.0) and sequential blocking in 0.3% hydrogen peroxide and 20% rabbit serum (Dako), before incubation with primary antibodies: mouse anti-rat CD8 (Serotec; dilution 1:100) and mouse anti-rat ED-1 (Serotec; dilution 1:500) at room temperature for 1 hour. After washing, slides were incubated with rabbit anti-mouse immunoglobulin secondary Ab (Dako; dilution 1:100) for 1 hour at room temperature, washed again, and incubated with HRP-conjugated Extravadin (Sigma; dilution 1:100) for 30 minutes, before final development in DAB. Sections were counterstained and mounted as described previously. CD8 positive cell infiltrate was quantified by a blinded observer by counting the number of CD8 positive cells in 50 consecutive glomeruli in each section, and expressed as the mean number of cells per glomerular cross section for each animal. ED1 positive cell infiltrate was quantified by a blinded observer using automated image analysis software (ImagePro Plus) to measure the percentage of glomerular staining in 20 consecutive glomeruli in each section, and expressed as the mean percentage for each animal.

**Assay for α3(IV)NC1 antibodies**

The concentration of anti-α3(IV)NC1 antibodies in rat sera was measured by enzyme linked immunosorbent assay (ELISA). Recombinant rat α3(IV)NC1 was diluted in 5mM carbonate buffer (pH 9.5) and coated onto 96 well ELISA plates (Nunc, Roskilde, Denmark) at a concentration of 5µg/well. The plates were left overnight at 4°C, and then washed by 0.1%Tween/PBS three times. 3% bovine serum albumin (BSA; Sigma) was then applied and the plate incubated for 1 hour at 37°C, followed by another series of washing. Rat sera were diluted in PBS, and added to wells in triplicate. The plate was incubated for one hour at 37°C, followed by a series of washing. An ALP-conjugated goat anti-rat IgG (Sigma) diluted 1:1000 in PBS was added to each well, and the plate incubated for one hour at 37°C, followed
by a series of washing. The plate was then developed using p-nitrophynyl phosphate solution
(Sigma) and read at 405nm using an ELISA plate reader (Biotech EL800, Biotech
Instruments Ltd., UK). Sera with a high titre of anti-α3(IV)NC1 antibodies was serially
diluted and used to develop a one-site binding hyperbole standard curve, from which results
were interpolated and expressed in arbitrary units.

**Direct immunofluorescence for deposited α3(IV)NC1 antibodies and complement C3**

IgG and C3 deposited in the glomeruli was assessed by direct immunofluorescence using
snap frozen renal tissue obtained at the time of cull by placing kidney tissue on cork-boards,
covering with OCT embedding medium (Thermo Scientific), immersing in isopentane, and
cooling in liquid nitrogen before storing at -80ºC. Sections were subsequently cut on a
cryostat at 5µm thickness and placed on poly-L-Lysine coated slides. After fixation in
acetone for 10 minutes, the slides were air dried. The slides were blocked with 20% normal
rabbit serum (Dako) for 30 minutes at room temperature. After washing in PBS, the slides
were incubated with FITC-conjugated rabbit anti-rat IgG (Sigma) at 1:100 dilution in PBS, or
with FITC-conjugated goat anti-rat C3 (Nordic MUbio, Susteren, The Netherlands) at 1:10
dilution, for one hour at room temperature. Following two ten minute washes in PBS, the
sections were mounted in PBS/Glycerol (Citifluor, UK). For quantification, 20 consecutive
glomeruli on each section were inspected by a blinded observer, and the degree of linear
immunofluorescence graded from 0 to 3+, with results expressed as the mean intensity per
glomerulus for each animal.
B cell ELISpot assays

Splenocytes were obtained from animals after oral gavage treatment with fostamatinib or vehicle formulation, either from day 0-18 or from day 18-36 after disease induction. In total, three pairs of animals were used for each time-point, and representative replicate results from one pair are shown in the manuscript. Splenocytes were obtained by passing whole spleen tissue through 100µm filters in cold sterile PBS. Following red cell lysis in hypotonic saline (NH₄Cl, NaHCO₃, EDTA, H₂O), and three washing-resuspension cycles in sterile PBS, the cells were resuspended in RPMI (Invitrogen, Paisley UK), 10% fetal calf serum (FCS; MB Meldrum Ltd., UK) 5000iu/ml penicillin (Invitrogen), 5000µg/ml streptomycin (Invitrogen), 2mM L-glutamine (Invitrogen) to a concentration of 5 million cells/ml. 100ul of cell suspension (containing 500,000 cells; 8 replicates per animal) was then added to each of well of ELISpot plates (Multiscreen HTS 96 well filter plates; Millipore, Billerica MA) that had previously been coated with recombinant rat α3(IV)NC1 at a concentration of 50µg/ml in sterile PBS, and blocked in 10% FCS/RMFI. Cells were then incubated at 37°C for 48 hours without moving. After washing, the ELISpot plates were incubated with a biotinylated polyclonal rabbit anti-rat immunoglobulin secondary antibody (Dako; dilution 1:250) at room temperature for 2 hours. Following a further wash step, the plates were incubated with ALP-conjugated Extravidin (Sigma; dilution 1:1000) for 1 hour at room temperature, washed again, and finally developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) solution (Sigma). Membranes were dried, and the number of spots in each well, corresponding to antigen-specific antibody producing cells, was quantified using an ELISpot platereader and dedicated software (ELISpot 4.0, Autoimmun Diagnostika, Strassberg Germany).
Flow Cytometry

Flow cytometric analysis was performed on splenocytes obtained from animals after oral gavage treatment with either fostamatinib or vehicle formulation from day 0-18 after disease induction (n=4 per group). Spleens were harvested and mechanically dissociated through a 100μm cell-strainer. Erythrocytes were lysed in hypotonic saline (NH₄Cl, NaHCO₃, EDTA, H₂O) and the splenocytes resuspended in PBS prior to staining with the following antibodies: CD45RA-PE (OX-33; Becton Dickinson (BD) Biosciences, San Diego CA); CD8-PECy7 (OX8; eBiosciences); CD4-FITC (W3/25; eBiosciences, San Diego CA). Cells were washed, resuspended in PBS and run on a BD Accuri C6 flow cytometer. Analysis was performed on FlowJo X software (Tree Star, Ashland OR).

Ex vivo Glomeruli

Glomeruli were extracted from untreated animals 28 days after induction of EAG, by differential sieving of whole kidney tissue, using 250μm, 125μm and 75μm sieves. After 3 wash-resuspension cycles in cold PBS, glomeruli were resuspended in cell culture media (RPMI, 5000iu/ml penicillin, 5000μg/ml streptomycin, 2mM L-glutamine, all Invitrogen; and 10% FCS, MB Meldrum Ltd). All glomeruli obtained from a single rat were then divided in equal proportion to wells of 24-well cell culture dishes (Corning Incorporated, Corning NY) in the following conditions at a final volume of 500μl: vehicle (0.1% DMSO in full culture media), and 0.2μM, 1μM and 2μM R406 in vehicle. Glomeruli were incubated for 48 hours and culture media collected for analysis of cytokine levels, as described below. Since the yield of glomeruli from each rat was variable, results were normalised to vehicle conditions,
to allow comparison of each biological replicate. Results from 4 rats are shown in the manuscript.

**Bone Marrow Derived Macrophages (BMDM)**

BMDM were prepared by incubating bone marrow cells in L929 cell-conditioned media until day 7, in non-treated cell culture dishes (Sterilin, Newport UK). Following non-enzymatic dissociation (Biological Industries, Beit Haemek, Israel), cells were re-plated to 96-well culture dishes (Corning) at a density of 100,000 cells per well and serum-starved overnight. Prior to stimulation, cells were pre-incubated with vehicle (0.1% DMSO in serum-free culture media) or 0.2μM, 1μM and 2μM R406 in vehicle for 30 minutes. Whole rat IgG (Sigma) was dissolved in sterile saline, heat-aggregated by heating to 63°C for 20mins (as previously described), reconstituted to a final volume of 250mcg/ml in serum-free cell culture media, and used to stimulate BMDM. Cell culture supernatants were collected after 24 hours and cytokine levels assayed, as described below. Technical replicate (x3) results, from one of at least two biological replicate experiments, are shown in the manuscript.

**Cytokine assays**

We used the following reagents to quantify cytokine levels in glomerular and BMDM cell culture supernatants, in accordance with the manufacturer’s instructions: OptEIA Rat MCP-1 ELISA Set, and Cytometric Bead Array Rat TNF-α Flex Set, both BD Biosciences; Rat IL-12+p40 ELISA Kit, Invitrogen, Paisley UK)
Statistics

Statistical analysis was conducted using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA). All data reported as median ± interquartile range. Comparison between groups was by Mann-Whitney $U$ test and Kruskal-Wallis test with Dunn’s multiple comparison, for non-parametric data.

References for Supplementary Methods


