Characterisation of an enhanced preclinical model of experimental MPO-ANCA autoimmune vasculitis

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Abstract

Experimental autoimmune vasculitis (EAV) is a model of antineutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) induced by immunisation of susceptible rat strains with myeloperoxidase (MPO). Animals develop circulating MPO-ANCA, pulmonary haemorrhage and glomerulonephritis, although renal injury is mild and recovers spontaneously without treatment. In this study we aimed to augment the severity of glomerulonephritis. Following induction of EAV on day 0, a sub-nephritogenic dose of nephrotoxic serum (NTS) containing heterologous antibodies to glomerular basement membrane was administered on day 14. This resulted in a significant increase in disease severity at day 28 compared to MPO immunisation alone - with more urinary abnormalities, infiltrating glomerular leucocytes, and crescent formation that progressed to glomerular and tubulointerstitial scarring by day 56, recapitulating important features of human disease. Importantly, the glomerulonephritis remained pauci-immune, and was strictly dependent on the presence of autoimmunity to MPO, as there was no evidence of renal disease following administration of sub-nephritogenic NTS alone or after immunisation with a control protein in place of MPO. Detailed phenotyping of glomerular leucocytes identified an early infiltrate of non-classical monocytes following NTS administration that, in the presence of autoimmunity to MPO, may initiate the subsequent influx of classical monocytes which augment glomerular injury. We also showed that this model can be used to test novel therapeutics by using a small molecule kinase inhibitor (fostamatinib) that rapidly attenuated both glomerular and pulmonary injury over a four-day treatment period. We believe that this enhanced model of MPO-AAV will prove useful for the study of glomerular leucocyte behaviour and novel therapeutics in AAV in the future.

Keywords: MPO, ANCA, vasculitis, monocytes, glomerulonephritis, experimental vasculitis
Introduction

Anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis (AAV) is a rare systemic autoimmune disease, which can cause life-threatening lung haemorrhage and end stage kidney disease (ESKD) [1]. The typical renal lesion in AAV is pauci-immune crescentic glomerulonephritis. Circulating ANCA are directed to myeloperoxidase (MPO) or proteinase-3 (PR3), which are present in the granules of neutrophils and lysosomes of monocytes [2,3], and a number of experimental and clinical observations indicate they have a directly pathogenic role in disease pathogenesis [4,5].

Studies in animal models have been critical for understanding disease mechanisms, and several rodent models of anti-MPO vasculitis have been developed [6]. These include passive transfer of anti-MPO antibodies, raised in MPO-deficient mice, to naïve wild-type mice, causing glomerulonephritis (GN) and pulmonary capillaritis [5–7]. Models of active autoimmunity in mice have also been developed; mice immunised with mouse MPO develop anti-MPO antibodies at low titre, but these are not sufficient to cause GN, and a ‘second-hit’ is required to induce disease. For example, a subsequent injection of heterologous anti-mouse glomerular basement membrane (GBM) globulin results in transient neutrophil recruitment to the glomerulus. This ‘planting’ of the MPO autoantigen (derived from retained neutrophils) results in recruitment of MPO-specific CD4+ T cells, neutrophils and macrophages to the glomerulus, and the development of crescentic GN [8,9]. The disease triggered by anti-GBM globulin is dependent on MPO; disease does not occur in MPO-deficient mice. A limitation of this model is that the response to anti-GBM globulin is itself nephritogenic; by 4 days mice develop an autologous immune response to deposited anti-GBM antibody, leading to severe GN, even in the absence of pre-immunisation with MPO. As such, study of anti-MPO mediated disease is limited to early time points, meaning that therapeutics can only be tested in preventative studies, not after the development of vasculitis [9,10].
A model of experimental autoimmune vasculitis (EAV) in the susceptible Wistar–Kyoto (WKY) rat strain was previously described by Little et al in our laboratory. Rats immunised with human MPO develop polyclonal MPO-ANCA cross-reactive to rat MPO expressed in neutrophils and monocytes, and subsequently small vessel vasculitis, pauci-immune GN and haemorrhagic pulmonary capillaritis [11–14]. A limitation of the model is that renal disease is relatively mild; glomerular lesions are mainly proliferative, crescent formation is rare, and disease spontaneously resolves from six weeks post-induction. It has been shown in other studies in rats that addition of a low-dose of NTS, containing heterologous anti-rat GBM antibodies, after immunisation with MPO, results in increased disease severity [15,16], similar to the approach described in mice. However, this approach is not well characterised in rats, and in these reports the administration of NTS alone, in the absence of autoimmunity to MPO, induced GN, and immunoglobulin deposits could be detected in glomeruli.

In this study, we aim to augment disease severity of EAV by the addition of a truly sub-nephritogenic dose of NTS that is insufficient to induce disease in the absence of autoimmunity to MPO. To investigate possible mechanisms by which sub-nephritogenic NTS increases disease severity, we isolate and phenotype glomerular leucocytes using flow cytometry. Finally, we show that an enhanced model can be used to test therapeutic approaches in a preclinical study.
Materials and methods

Animal husbandry

WKY and Lewis rats were purchased from Charles River (Saffron Walden, UK) and maintained in a pathogen-free animal facility at the Central Biomedical Services unit, Hammersmith Hospital Campus, Imperial College London. All procedures were carried out in accordance with the regulations of the UK Animals (Scientific Procedures) Act (1986) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Experimental autoimmune vasculitis/NTS administration

NTS was prepared in rabbits as previously described [17]. For the NTS titration experiment, male WKY rats aged 8–9 weeks (n=4/group) were immunised intravenously (IV) with 100 µl of NTS (or serial dilutions in sterile PBS) and maintained until 10 days post-immunisation.

EAV was induced by immunising 7–8 week old male WKY or Lewis rats (n=4–10/group) intramuscularly with 800 µg/kg human MPO (Calbiochem, Merck Millipore, Darmstadt, Germany), or human serum albumin (HSA; Sigma, Poole, UK) as a control human protein, emulsified in complete Freund’s adjuvant supplemented with Mycobacterium butyricum (Sigma). Intraperitoneal pertussis toxin (Sigma) was administered on days 0 and 2 [13].

For ‘double’ immunisation studies, on day 14 after initial immunisation with MPO, 100 µl of 1:100 dilution of NTS or normal rabbit serum (NRS) was administered IV. Urine was collected weekly by housing animals in individual metabolism cages overnight, and blood collected every 14 days by lateral tail vein bleed. Animals were sacrificed after 28, 42 or 56 days.
For additional experiments to assess glomerular cell infiltrate at early time points, neat or 1:100 NTS was given IV to 8–9 week old male WKY rats (n=4/group) and animals sacrificed after 3 h, 24 h or 7 days.

**Assessment of renal disease**

Proteinuria was measured by the sulphosalicylic acid method and haematuria by urine dipstick (Multistix 8; Siemens, Munich, Germany) [18]. At the end of each experiment animals were exsanguinated under terminal anaesthesia. Renal tissue was fixed in 10% neutral buffered formalin, transferred to 70% ethanol, and processed to paraffin blocks. Sections were stained with periodic acid Schiff (PAS), haematoxylin and eosin, and Jones’ silver stain. Fifty consecutive glomeruli were assessed for crescents ± necrosis, segmental necrosis, or minor changes such as segmental endocapillary proliferation, by a blinded observer, and results expressed as mean proportion of glomeruli for each animal. Immunoperoxidase staining was carried out using CD68 (ED-1, Bio-Rad, Watford, UK; dilution 1:500), CD8 (OX-8, Bio-Rad; dilution 1:50), CD3 (1F4, Bio-Rad; dilution 1:400), and for smooth muscle actin (M0851, Dako, Ely, UK; dilution 1:100). Number of positive cells was quantified using ImageProPlus software to measure percentage area staining in 20 consecutive glomeruli, or 20 consecutive high-powered field (HPF) for interstitial staining, and results expressed as mean percentage for each animal. Smooth muscle actin immunostaining was used to quantify the area proportion of fibrous/fibrocellular crescents using assessment of 50 consecutive glomeruli.

Glomeruli were isolated by serial sieving of whole kidney tissue as described previously [19], then digested with 1 mg/ml Type IV collagenase (Sigma), 0.5 mg/ml trypsin (Sigma) and 0.1 mg/ml Type I DNase (Roche, Welwyn, UK) for 20 min at 37 °C with gentle agitation. Cells were washed and used for cell surface staining with antibodies against CD172a (OX-41, FITC, Biorad, 1:5), CD45 (OX-1, V450, BD Biosciences, Oxford, UK, 1:40), CD3 (eBioG4.18, PE,
eBiosciences, Hatfield, UK, 1:40), B220 (HIS24, PE, eBiosciences, 1:40), CD161a (3.2.3, PE, BioLegend, San Diego, USA, 1:40), granulocyte marker antibody (HIS48, biotin, eBiosciences, 1:40) and CD43 (W3/13, AlexaFluor647, BioLegend, 1:40) followed by streptavidin-PECy7 (BioLegend, 1:300) secondary. Cells were analysed on a BD LSRFortessa flow cytometer with standard lasers, and gating strategy and analysis performed using FlowJo v10 software [20]. For quantification of cell numbers, precision count beads (BioLegend) were used.

**Assessment of lung injury**

Severity of lung injury was graded by visual inspection of the lungs using a semi-quantitative scoring system which graded lungs as: 0- normal; 1- less than 10 petechiae; 2- 10–20 petechiae; and 3 if > 20 petechiae were seen. Lung tissue was also collected and processed as for kidney tissue. Perls’ Prussian blue staining was used to quantify haemosiderin-laden macrophages using ImageProPlus software by measuring proportion of stained cells in 5 HPF.

**Assessment of autoantibody response**

Anti-MPO antibodies were assayed in serum using a direct ELISA. Plates were coated with 1.33μ g/ml of hMPO overnight, blocked with 3% BSA followed by rat serum, and standards diluted in PBS. A goat anti-rat IgG-ALP conjugate (1:1000, Sigma) was used as a secondary antibody and plates were developed with p-nitrophenyl-phosphate solution (Sigma).

IgG binding to rat leucocytes was assayed using flow cytometry and indirect immunofluorescence (IIF). For flow cytometry, whole blood was collected via cardiac puncture, red cells were lysed (1X RBC Lysis Buffer, eBioscience) and cells were used for cell surface staining with antibodies against CD3 (eBioG4.18, PE), B220 (HIS24, PE), and CD161a (3.2.3, PE), all 1:40. Cells were fixed and permeabilised then incubated with 1:1000 dilution of rat serum followed by an Alexa647 conjugated anti-rat IgG secondary (1:1000, Biolegend).
Cells were analysed on a BD LSRFortessa flow cytometer with standard lasers, and gating strategy and analysis performed using FlowJo v10 software. The mean fluorescence intensity (MFI) of PE-negative cells was used to quantify IgG binding. For IIF rat bone marrow was applied to microscope slides using a cytopsin at 300 rpm for 3 min. Cells were fixed in 95% ice cold ethanol for 10 min, blocked for 20 min in 20% goat serum and incubated with rat serum diluted 1:10 in PBS. Bound IgG was detected using FITC-conjugated goat anti-rat IgG (1:1000, Sigma).

Deposited rat and rabbit IgG was assessed using direct immunofluorescence on frozen kidney sections using FITC-labelled antibodies and quantified by examining 20 glomeruli and scoring each as 0 to 3+, with results expressed as mean per animal. Indirect immunofluorescence for C3 was carried out using anti-C3 (1:200, 12E2, Abcam) with anti-mouse FITC secondary (1:200, Vector Laboratories, Peterborough, UK). Direct immunofluorescence for MPO was carried out using anti-MPO FITC (Abcam, 2D4) and to aid visualisation of glomeruli, tomato-lectin-DyLight 594 conjugate was added (Vector).

For electron microscopy, renal tissue was collected in 2.5% glutaraldehyde. Processing of tissue sections and imaging was carried out by North West London Pathology.

**SYK inhibitors**

Fostamatinib disodium (R788) was a gift from Rigel Pharmaceuticals (South San Francisco, California). It was reconstituted in vehicle formulation (0.1% carboxymethylcellulose, 0.1% methylparaben sodium, 0.02% propylparaben sodium, in distilled water, pH 6.5). Based on a previous dose-ranging study in nephrotoxic nephritis (NTN) in WKY rats, animals received 30 mg/kg, administered by twice daily oral gavage [21]. Control animals received an equivalent volume and schedule of vehicle formulation.

**Statistical Analysis**
Statistical analysis was conducted using Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Unless otherwise stated, all data are reported as median with interquartile range. Where appropriate, Mann–Whitney U and Kruskal–Wallis tests were used to assess the difference between 2 or >2 groups, with Dunn’s *post hoc* test to compare individual groups.
Results

Administration of a sub-nephritogenic dose of NTS in EAV increases renal injury in the presence of autoimmunity to myeloperoxidase, and disease remains pauci-immune

In the conventional NTN model, WKY rats are immunised with 100 µl neat (undiluted) rabbit anti-rat NTS by intravenous (IV) injection, resulting in rapid glomerular deposition of rabbit IgG, urinary abnormalities by day 4, deposition of autologous rat IgG by day 6, and severe crescentic GN by day 10 [17].

To identify a sub-nephritogenic dose of NTS, rats were immunised with serial dilutions of NTS from neat to 1:100 (n=4 per group) and assessed at day 10. After immunisation with 1:50 or lower dilutions of NTS, there was no detectable glomerular injury, and there was no evidence of either deposited rabbit or autologous rat IgG within the kidney by direct immunofluorescence (supplementary material, Figure S1A–J). Thus, a 1:100 dilution of NTS was selected as a sub-nephritogenic dose for all subsequent experiments.

To assess whether the addition of this sub-nephritogenic dose of NTS could augment disease severity in EAV, WKY rats were immunised with human MPO (or human serum albumin (HSA) as control) on day 0, followed by 1:100 NTS (or 1:100 normal rabbit serum (NRS) as control) on day 14 (n=4–6/group). This time point (day 14) after MPO immunisation was selected because rats have developed circulating MPO-ANCA but no urinary abnormalities or glomerular injury. By 7 days after administration of NTS (21 days after MPO immunisation) animals began to develop urinary abnormalities. At day 28 after initial immunisation with MPO, the addition of 1:100 NTS caused significant increases in haematuria (Figure 1A; median dipstick haematuria 3,0,0,0 for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively, p<0.0001) and proteinuria (Figure 1B; median proteinuria 137.0, 5.0, 6.0, 3.4 mg/day for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively, p<0.0001).
Histological assessment at day 28 showed glomerular necrosis and crescents in all animals immunised with MPO/NTS, with around 60% abnormal glomeruli, including 30% with crescents. Disease was similar to that seen in AAV, with focal disease, crescents, and segmental necrosis the main features (Figure 1C,I). In animals immunised with MPO/NRS there were mild proliferative changes and occasional crescent formation, in keeping with early conventional EAV (supplementary material, Figure S2). Animals immunised with HSA and NTS/NRS had near normal glomerular histology (supplementary material, Figure S2). There was a significant increase in cell infiltration into glomeruli at 28 days in MPO/NTS animals when assessed by immunostaining and this was predominantly CD68/ED-1+ monocyte/macrophages (Figure 1D,I; median % staining/glomerular cross section (GCS) 5.65, 0.04, 0.12, 0.01 for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively, p<0.0001). A smaller infiltrate of CD8+ (Figure 1E,I; median % staining/glomerular cross section (GCS) 0.84, 0.01, 0.02, 0 for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively, p=0.005) and CD3+ (Figure 1F,I; median % staining/glomerular cross section (GCS) 0.92, 0.04, 0.08, 0.03 for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively, p=0.01) cells were also present. The glomerular cell infiltrate was further phenotyped using flow cytometry; this showed an increase in both non-classical and classical monocytes in animals immunised with MPO/NTS, and a small increase in non-classical (NC) monocytes in animals immunised with HSA/NTS (Figure 1G,H; median 17.4, 7.3 and 8.2 NC monocytes/glomerulus for MPO/NTS, MPO/NRS, HSA/NTS respectively, p=0.002).

Importantly, in animals immunised with sub-nephritogenic NTS, disease remained pauci-immune with no detection of deposited autologous rat IgG or C3 at day 28 in any group by indirect immunofluorescence (Figure 2A–F). There were no deposited immune complexes seen using electron microscopy in any group (Figure 2G,H). Cellular crescents and cells interacting with the GBM were seen by electron microscopy in animals immunised with
MPO/NTS, but not in other groups (Figure 2G,H and supplementary material, Figure S3A–D).

Susceptibility to GN in this model was limited to the WKY rat strain. Despite developing robust auto-immunity to MPO, Lewis rats did not develop urinary or glomerular abnormalities following immunisation with MPO and low-dose NTS (supplementary material, Figure S4A–F). Immunostaining for monocytes/macrophages using CD68/ED-1 identified a small cellular infiltrate (supplementary material, Figure S4G,H). This was also seen on flow cytometry phenotyping of glomerular cellular infiltrate which identified a small infiltrate of non-classical monocytes (supplementary material, Figure S4I).

**Addition of a sub-nephritogenic dose of NTS has no effect on lung injury or circulating autoantibodies**

There was no difference in lung haemorrhage severity in animals given 1:100 NTS in addition to MPO; both by visual inspection (Figure 3A; median lung haemorrhage score 1,1,0,0 for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively) and by Perls’ staining for haemosiderin-laden macrophages (Figure 3B,C; median Perls’ stain 0.13, 0.11, 0, 0 au for MPO/NTS, MPO/NRS, HSA/NTS, HSA/NRS respectively). The degree of lung injury was in keeping with that seen in our previous studies of EAV without additional NTS/NRS [14]. There was no difference in circulating MPO-ANCA levels between the groups of rats immunised with MPO either by ELISA using human MPO, or by flow cytometry to assess IgG binding to rat leucocytes (Figure 3D,E). IIF using normal rat bone marrow cells confirmed that sera from rat immunised with hMPO (+/-NTS) resulted in perinuclear staining in cells with neutrophil nuclear morphology (Figure 3F).

**Rats immunised with MPO followed by a sub-nephritogenic dose of NTS develop glomerular and tubulointerstitial scarring at 6 and 8 weeks**
In the conventional EAV model, disease spontaneously resolves beyond six weeks. To assess disease phenotype at later time points in this enhanced model, WKY rats (n=5 or 6/group) were immunised with the protocol described above for examination at days 42 and 56. We did not examine control animals (MPO/NRS, HSA/NTS, HSA/NRS) at these time points as no significant disease was present in these groups at 28 days. Proteinuria decreased steadily after day 28 in 10/11 rats. One animal developed persistent high levels of proteinuria until day 56 (Figure 4A; median proteinuria 69.9, 54.5, 55.8, 37.9 and 40.1 mg/day at day 28, 35, 42, 49 and 56, respectively). All rats continued to have 3+ haematuria until time of sacrifice (Figure 4B). Cellular crescents at day 28 progressed to a mixture of cellular and fibrocellular crescents at day 42, with further progression at day 56 (Figure 4C,H). Glomerular cell infiltrate decreased sequentially at days 42 and 56 (Figure 4D,E). CD68/ED-1+ cell infiltrate was predominately tubulointerstitial and peri-glomerular at both time points (Figure 4E,G; median glomerular CD68/ED-1 staining 1.0 and 0.6 %/GCS at week 6 and week 8, respectively).

Smooth muscle actin (SMA) staining was used to identify myofibroblasts and the development of fibrocellular crescents, with predominantly interstitial staining seen at day 42, and glomerular and interstitial staining by day 56 (Figure 4F,G,J; median tubulointerstitial staining 0.23, 2.7 and 6.1%/HPF at day 28, 42 and 56 respectively, p=0.0004; median glomerular staining 0.03, 1.2 and 10.1%/GCS at day 28, 42 and 56 respectively, p<0.0001).

There was continuing evidence of lung haemorrhage in most animals, more severe at day 42 than day 56, in keeping with the natural history of conventional EAV (supplementary material, Figure S5A–C; median Perls’ stain 1.4 and 0.4 at day 42 and 56 respectively). Circulating MPO-ANCA levels peaked at day 42 after immunisation (supplementary material, Figure S5D). There was no evidence of deposited glomerular rat or rabbit IgG at days 42 or 56.

Sub-nephritogenic NTS induces early infiltrate of non-classical monocytes
In the autoimmune MPO model of GN in mice, administration of NTS is thought to cause glomerular neutrophil infiltration and de-granulation, extracellular MPO deposition, and augmented glomerular injury in mice with autoimmunity to MPO [9,10]. To investigate this in our model, we re-examined early time points after administration of NTS. Rats were immunised with either neat or 1:100 NTS and sacrificed at 3 or 24 h (n=4/group). By direct immunofluorescence, there was strong linear deposition of rabbit IgG following immunisation with neat (undiluted) NTS and faint deposition of rabbit IgG within the kidney at 3 h with 1:100 NTS, which largely resolved by 24 h (Figure 5A). It was not possible, to quantify the difference in fluorescence intensity between this and neat NTS as the magnitude of the difference was so great that images could not be captured with comparable exposure times. Images of direct immunofluorescence for rabbit IgG in a normal rat, and after immunisation with 1:100 NRS with equivalent exposure times are shown for comparison (Figure 5A). At 3 h after immunisation with 1:100 NTS, there was patchy deposition of complement C3, of decreased intensity compared to animals immunised with neat NTS (Figure 5B).

We then examined glomerular cell infiltrate at these early time points using two methods (Figure 5C–E). By both indirect immunofluorescence and flow cytometry there was significant neutrophil infiltrate and deposition of granular extracellular MPO within glomeruli at 3 h after immunisation with neat NTS. This was not seen following immunisation with 1:100 NTS; neutrophil number in this group was similar to that in normal rats. The neutrophil infiltrate seen with neat NTS reduced by 24 h, suggesting a transient effect on neutrophil recruitment (Figure 5C; median neutrophils/glomerulus 10.5, 1.05, 1.3 and 0.8 for 3 h after neat NTS, 24 h after neat NTS, 3 h after 1:100 NTS and normal rats respectively. Figure 5D, E; median cells/GCS 2.3, 0.5, 0.15, 0.06 for 3 h after neat NTS, 24 h after neat NTS, 3 h after 1:100 NTS, and normal rats respectively).
Using flow cytometry, by 24 h after immunisation with 1:100 NTS there was a small infiltrate of non-classical monocytes, lower than that seen with neat NTS, but significantly higher than normal rats (Figure 5C; median 5.8, 4.9 and 7.7 cells per glomerulus for normal, 3 h and 24 h after 1:100 NTS respectively, p=0.03 for 24 h compared to normal). This suggests that a sub-nephritogenic dose of NTS may alter early non-classical monocyte, rather than neutrophil, retention within glomeruli in this model.

**Fostamatinib treatment reduces the severity of renal and lung injury in augmented EAV**

We next set out to identify whether this model was useful for evaluation of therapeutic strategies. We have previously used fostamatinib, a small molecule inhibitor of spleen tyrosine kinase (Syk), as treatment in experimental models of GN, including EAV [14,21]. Given the rapid onset of therapeutic effect in previous experiments, we elected to assess a short period of treatment in the present study. Animals were treated from day 24 (after onset of renal disease evidenced by urinary abnormalities) until day 28. In keeping with our previous studies, fostamatinib was administered at a dose of 30 mg/kg twice daily by oral gavage (n=5 or 6/group).

After 4 days treatment with fostamatinib, there was a significant reduction in proteinuria compared to vehicle-treated animals (Figure 6A; median proteinuria/day 62.8 mg and 9.4 mg for vehicle- and fostamatinib-treated rats respectively, p=0.002). There was a non-significant reduction in haematuria, but this was not seen in all animals, likely reflecting the short treatment period (Figure 6B; median dipstick 3 and 2.5 for vehicle- and fostamatinib-treated rats respectively). There was a significant improvement in glomerular injury, with near normal histology in fostamatinib-treated animals, and around 50% abnormal glomeruli in vehicle-treated rats (Figure 6C). Glomerular infiltrating leucocytes were reduced by both CD68/ED-1 immunohistochemistry (Figure 6D, median CD68/ED-1 % staining/GCS 4.2 and 0.02 for
vehicle and fostamatinib-treated rats respectively, p=0.004) and flow cytometry (Figure 6E). Fostamatinib also improved lung injury; compared to vehicle-treated animals, there were decreases in visual lung haemorrhage score (Figure 6F; median score 1.5 and 0 for vehicle and fostamatinib-treated rats respectively, p=0.04) and hemosiderin laden cells (Figure 6G; median Perls’ score 0.11 and 0 for vehicle and fostamatinib-treated rats respectively, p=0.01). As expected with a short duration of treatment, there was no difference in circulating MPO-ANCA levels (Figure 6H).

Discussion

We have shown that adding a sub-nephritogenic dose of NTS to immunisation with MPO results in significant augmentation of renal injury in EAV. By using a low-dose of NTS, this model remains pauci-immune with no detectable immune complex deposition by conventional methods (either immunofluorescence or electron microscopy) at the time of crescentic GN. The model remains critically dependent on the presence of autoimmunity to MPO, as disease does not occur following immunisation with a control human protein.

In contrast to previously described rodent models, an autologous response to NTS does not occur at any time point in this model [9,22–24]. In a previous study in the Brown Norway rat, which is less susceptible to GN than the WKY strain, animals immunised with low-dose NTS, even in the absence of autoimmunity to MPO, developed clear evidence of GN, including proteinuria and glomerular macrophage infiltration. In addition, disease was not pauci-immune, as strong linear deposits of rabbit IgG were seen at all time points, with deposited rat IgG in areas of crescent formation [16]. Another study used the susceptible WKY strain, as in our model. Again, however, the dose of NTS in isolation caused overt GN with substantial haematuria and 5% glomerular crescents by light microscopy, and no data were provided regarding the pattern of glomerular immunoglobulin deposition [15]. By contrast, in our study,
the dose of NTS has been decreased such that no urinary or glomerular abnormalities occur in rats immunised with low-dose NTS following a control protein.

This model has several advantages over the conventional EAV protocol; it allows for a shortened disease course from 42 to 28 days and, as disease is more reproducible and of greater severity, for a reduction in the number of rats needed to test therapeutic approaches. Rather than complete resolution after day 28, disease progresses to fibrosis and scarring, which more accurately recapitulates the natural history of ANCA-associated GN in humans. Disease is somewhat heterogenous, particularly the degree of proteinuria (which ranged from 36–220 mg/day). Glomerular crescents and CD68/ED-1+ cell infiltration were less variable; these may be better outcome measures to use in studies of therapeutic approaches.

Analysis of glomerular infiltrating cells at day 28 showed that a 1:100 NTS dose alone induced a small influx of non-classical monocytes (median 8.2 and 6 cells/glomerulus in animals immunised with HSA/NTS and normal WKY rats respectively, p=0.1) but in the presence of autoimmunity to MPO this was much greater (17.3 cells/glomerulus, p=0.0005) and accompanied by a significant infiltrate of classical monocytes. Although we could not detect immune complexes or deposited autologous IgG within the kidney, low-dose NTS is clearly mediating a biological effect. We show that within 3 h of injection it is transiently localised to the kidney at very low levels, accompanied by C3 deposition, and this is followed by a small but significant infiltration of non-classical monocytes by 24 h. We hypothesise that this influx of non-classical, patrolling monocytes occurs in response to low-dose NTS in glomeruli, via direct Fc receptor- (CD16-) or complement-dependent mechanisms. This is in keeping with intravital microscopy studies in both mice and rats with NTN [20,25–28]. In WKY rats, we have recently shown that in response to nephritogenic stimuli, there is increased LFA-1-dependent surveillance of the endothelium by non-classical monocytes, and subsequent classical monocyte retention within the glomerulus in conventional NTN [20]. We now show
that this response is insufficient to initiate GN after a reduced dose of NTS alone. However, these non-classical monocytes express MPO, and we hypothesise an interaction with circulating MPO-ANCA in MPO-immune rats – such that these retained and activated non-classical monocytes may initiate disease and promote classical monocyte recruitment.

In a well-characterised mouse model of MPO-AAV, low-dose NTS is thought to mediate its actions by recruiting neutrophils to the glomerulus. In our model we could not detect early infiltration of neutrophils following a sub-nephritogenic dose of NTS. This does not exclude a role for neutrophils in this cell type, as neutrophil survival may be impaired during tissue processing, and may lead to an under-estimate of their true number in our flow cytometric analysis. Future studies using intravital microscopy and live cell imaging will be useful to definitively assess early glomerular cell infiltrate following injection of neat and 1:100 NTS.

Mechanisms may also differ between species and rodent strains. The WKY rat has a pro-inflammatory monocyte/macrophage phenotype, such that disease in this strain may be dependent on these cell types [29]. In keeping with this, we show that although Lewis rats develop a small glomerular infiltrate of non-classical monocytes at day 28 after immunisation with MPO and low dose NTS, they do not develop features of GN, again suggesting that monocyte/macrophage responses contribute disease initiation in this model. This may have relevance for clinical translation, as there is increasing evidence that monocytes play a previously under-appreciated role in AAV. Monocytes express the ANCA autoantigens, stimulation with ANCA in vitro leads to cytokine production and generation of ROS, and monocytes and macrophages are the predominant cell types identified in renal biopsies from patients with AAV. As such, a monocyte/macrophage dependent mechanism in this model may be in keeping with aspects of disease pathogenesis in humans.
Finally, we also showed that this model is useful for testing therapeutic approaches, by administering a Syk inhibitor during established disease. Notably, in the present study we used a very short treatment period of only 4 days and show rapid reversal of glomerular injury, with a significant effect on infiltrating monocytes. These data support clinical investigation of Syk inhibition as a therapeutic target.

By adding a sub-nephritogenic dose of NTS to the existing model of EAV, we have developed a model of MPO-AAV which has several advantages over the standard protocol. It is more reproducible, with a greater proportion of crescents and more severe glomerular damage. This allows for a reduction in the number of animals required when testing therapeutic agents and, by timing of administration of therapeutic interventions, it is possible for investigators to distinguish their effects on both autoimmunity and glomerular injury. Unlike standard EAV, this model progresses to scarring and fibrosis, meaning it can also be used to study endpoints relevant to clinical disease. Crucially, disease remains pauci-immune and critically dependent on the presence of autoimmunity to MPO. We believe this model complements existing in vivo approaches for investigating AAV, and that it will prove valuable for future preclinical therapeutic studies.

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Author contributions statement

MP: conceptualisation, investigation, methodology, writing-original draft. KG: Investigation, writing (review and editing). TTS: Investigation, methodology, writing (review and editing). GB: Investigation. DC: Investigation; KW: Resources, methodology, writing (review and editing). HTC: conceptualisation, writing (review and editing), FWKT: conceptualisation, writing (review and editing). CR: conceptualisation, investigation, writing (review and editing). CDP: conceptualisation, investigation, writing- (review and editing). SPM: conceptualisation, investigation, writing (original draft).
Figure Legends

Figure 1. Addition of a sub-nephritogenic dose of NTS increases renal injury in the presence of autoimmunity to myeloperoxidase

When animals were immunised with 1:100 NTS at day 14 after hMPO there was a significant increase in (A) haematuria and (B) proteinuria by day 28. (C) Quantification of glomerular histology showed significant increase glomeruli with crescents and/or necrosis in animals immunised with MPO/NTS. (D) Quantification of CD68/ED-1 immunoperoxidase staining. (E) Quantification of CD8 immunoperoxidase staining. (F) Quantification of CD3 immunoperoxidase staining. (G) Gating strategy for neutrophils and monocyte subsets infiltrating glomeruli. Myeloid cells are identified as CD172a^Lin^ within CD45^+^ leukocyte gate. Neutrophils were differentiated from monocytes based on their CD172a and HIS48 expression. Neutrophils are identified as CD172a^hi^HIS48^hi^. Monocytes are identified as CD172a^hi^, and CD43 and HIS48 expression used to differentiate CD43^hi^HIS48^int^ non-classical monocytes and CD43^lo^HIS48^hi^ classical monocytes. (H) Glomerular infiltrating cells showing significant infiltrate of non-classical (NC) and classical monocytes at day 28 in animals immunised with MPO/NTS. (I) Representative images of glomerular pathology in animals immunised with MPO/NTS showing the presence of crescents and necrosis on H&E, PAS), and Jones’ silver stain. CD68/ED-1+ and CD3+ cells are mainly found in areas of crescent formation and CD8+ cells are scattered throughout glomeruli. Original magnification of images x400. Immunoperoxidase staining shown with haematoxylin counterstain. Images of histopathology from control groups is shown in supplementary material, Figure S2.

All data are shown as median with IQR. Kruskal–Wallis test with Dunn’s post hoc correction to MPO/NRS group *p<0.05, **p<0.01 ***p<0.001. NTS- Nephrotoxic serum, NRS- Normal
rabbit serum, HSA- human serum albumin. MPO- myeloperoxidase, no- neutrophil, mo- monocyte

**Figure 2. Sub-nephritogenic NTS does not result in glomerular immune complex deposition**

(A) Quantification of direct immunofluorescence for deposited rabbit IgG. (B) Representative images. Top panel shows no deposited IgG in MPO/NTS immunised rat and bottom panel representative image after immunisation with neat NTS. (C) Quantification of direct immunofluorescence for deposited rat IgG. (D) Representative images. Top panel shows no deposited IgG in MPO/NTS immunised rat and bottom panel representative image after immunisation with neat NTS. (E) Quantification of indirect immunofluorescence for deposited rat C3 (F) Representative images. Top panel shows no deposited C3 in MPO/NTS immunised rat and bottom panel representative image after immunisation with neat NTS. (G,H) Representative electron microscopy images from rats immunised with MPO/NTS showing no immune complex deposition. (G) Evidence of cellular crescent formation (black arrow indicates GBM, white arrow Bowman’s capsule. Cells and inflammatory material are seen in Bowman’s space) in an animal immunised with MPO/NTS; and (H) a monocyte (black arrow) directly interacting with the GBM (white arrow). IF images original magnification x400.

Images/quantification in experimental animals were obtained at day 28 (14 days after immunisation with NTS). Images for comparison following neat NTS administration were obtained at day 14 after immunisation with NTS (without prior immunisation with MPO).

Electron microscopy images from control groups are shown in supplementary material, Figure S3. NTS- Nephrotoxic serum NRS- Normal rabbit serum
Figure 3. Addition of a sub-nephritogenic dose of NTS has no effect on lung injury or autoimmunity to MPO

(A) Most animals immunised with MPO developed evidence of lung haemorrhage by visual inspection, regardless of second immunisation with NTS or NRS. (B) Quantification of lung haemorrhage using Perls’ Prussian Blue stain for haemosiderin laden macrophages. (C) Representative images shown for animals immunised with MPO or with HSA (without counterstain; original magnification x200). (D) Circulating anti-MPO titres, and (E) quantification of sera binding to permeabilised rat leucocytes, showing no difference in animals immunised with MPO regardless of the addition of NTS or NRS. (F) Representative images of indirect immunofluorescence rat bone marrow cells with diluted rat sera and anti-rat IgG FITC. Perinuclear staining is seen in cells with typical neutrophil nuclear morphology with sera from rats immunised with MPO but not HSA. Original magnification of images x400. NTS-Nephrotoxic serum NRS- Normal rabbit serum MPO- myeloperoxidase HSA-human serum albumin

Figure 4. Immunisation with MPO and NTS results in glomerular scarring at day 42 and 56

(A) In 10 of 11 rats, proteinuria plateaued at day 28–35 and then decreased steadily. In one animal (included in the total group but also represented separately as ‘outlier’) proteinuria continued to increase to day 56. (B) Haematuria remained at 3+ in all rats from day 28 for the duration of the experiment. (C) Quantification of fibrocellular and fibrous glomerular crescents at day 28, 42, and 56. (D) Quantification of glomerular infiltrating leucocytes by flow cytometry, showing decreased infiltrating cells at these time points with a return to near-normal at day 56. (E) Quantification of CD68/ED-1+ cells infiltrating glomeruli at day 42 and 56 after disease induction, showing a progressive decrease from day 42 to 56. (F) Quantification of
glomerular smooth muscle actin (SMA) staining at 28, 42 and 56 days showing a small increase by day 42 and significant increase at day 56. (G) Quantification of interstitial SMA staining at 28, 42 and 56 days showing a small increase by day 42 and significant increase at day 56. (H) Representative photomicrographs of PAS and Jones’ silver stained sections of renal tissue, showing development of fibrocellular crescents and peri-glomerular inflammation at day 42, and further progression of these changes by day 56. (I) Representative photomicrographs of CD68/ED-1 staining, showing mainly tubulointerstitial and peri-glomerular cell infiltrate at day 42 and 56. (J) Representative photomicrographs of SMA staining showing the development of interstitial staining at day 42 and profound interstitial, staining with development of fibrous crescents at day 56. Where appropriate, day 28 results are shown for comparison. All data are shown as median with IQR. Original magnification of images x400 (glomeruli) x200 (tubulointerstitium). Kruskal–Wallis test with Dunn’s post hoc correction *p<0.05, **p<0.01 ***p<0.001. NC- Non-classical

**Figure 5. Immunisation with 1:100 NTS results in an early increase in non-classical monocyte recruitment to glomeruli, without a detectable increase in neutrophils**

(A) Direct immunofluorescence for deposited rabbit IgG at 3 and 24 h after immunisation with neat and 1:100 NTS. A representative image of a normal rat, and a rat immunised with 1:100 NRS are shown for comparison. (B) Indirect immunofluorescence for deposited C3 at 3 and 24 h after immunisation with neat and 1:100 NTS. (C) Quantification of direct immunofluorescence for myeloperoxidase (MPO), with representative images in (D; original magnification x400). Arrow indicates MPO+ cells with typical neutrophil nuclear morphology in the glomerulus of a rat immunised with neat NTS at 3 h. No neutrophils are seen with animals immunised with 1:100 NTS. (E) Cell count of glomerular infiltrating cells at 3 and 24 h after immunisation with neat or 1:100 NTS, showing neutrophil infiltrate at 3 h in animals
immunised with neat but not 1:100 NTS, and a small infiltrate of non-classical monocytes at 24 h after immunisation with 1:100 NTS. All data are shown as median with IQR. Kruskal–Wallis test with Dunn’s post hoc correction *p<0.05, **p<0.01 ***p<0.001. NC- non classical, NTS- nephrotoxic serum; GCS, glomerular cross section

**Figure 6. Fostamatinib treatment decreases renal and lung injury in rats with enhanced EAV**

(A) Proteinuria and (B) haematuria from disease induction to day 28, with the fostamatinib treatment period shaded in grey, showing significant reduction in proteinuria in the fostamatinib-treated group. (C) Quantification of glomerular abnormalities at day 28 after disease induction showing minimal glomerular abnormalities in fostamatinib-treated rats. (D) Quantification of CD68/ED-1+ cells infiltrating glomeruli at day 28, with almost no cellular infiltrate in fostamatinib-treated rats. (E) Glomerular infiltrating leucocytes, showing significant decrease in classical and non-classical monocytes in the fostamatinib-treated group. (F) Significant decrease in lung haemorrhage in fostamatinib-treated rats by visual inspection and (G) Perls Prussian blue staining for haemosiderin laden macrophages. (H) Circulating anti-MPO antibody titres were not different between fostamatinib- and vehicle-treated rats. Data are shown as median with IQR. Mann–Whitney test *p<0.05, **p<0.01. GCS, glomerular cross section
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