Therapeutic myeloperoxidase inhibition attenuates neutrophil activation, ANCA-mediated endothelial damage and crescentic glomerulonephritis

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Running title: MPO-inhibition in glomerulonephritis

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Significance statement

Free myeloperoxidase (MPO) is deposited in the glomeruli in various forms of crescentic glomerulonephritis (CGN) and is elevated in ANCA-associated CGN, which is the most common form of CGN in adults. MPO mediates neutrophil degranulation, neutrophil extracellular trap formation and contributes to ANCA-mediated endothelial damage. It is critically implicated in CGN pathogenesis and all of these pathways are significantly attenuated with use of a novel MPO inhibitor, AZM198, which also reduces disease severity in a preclinical CGN model. These data suggest that clinical MPO inhibition may represent a novel therapeutic strategy for diverse forms of CGN.

Abstract

Background: Myeloperoxidase (MPO) released following neutrophil and monocyte activation can generate reactive oxygen species leading to host tissue damage. Extracellular glomerular MPO deposition, seen in ANCA- associated vasculitis (AAV), may enhance crescentic glomerulonephritis (CGN) through antigen-specific T and B cell activation. MPO-deficient animals have attenuated glomerulonephritis early on, but augmented T cell responses. We investigated the impact of MPO inhibition, using the MPO inhibitor AZM198, to understand its potential role in treating CGN.

Methods: Renal biopsies with various forms of CGN were stained for MPO and neutrophils (CD15). Serum MPO concentration was measured in AAV patients and healthy controls, while MPO activity, ROS production and neutrophil degranulation was assessed in ANCA-stimulated-neutrophils in the absence and presence of AZM198. We tested the effect of AZM198 on TNFα-primed ANCA-stimulated neutrophil-mediated endothelial cell (EC) damage *in vitro* and on CGN severity and antigen-specific T cell reactivity *in vivo*, using the murine model of nephrotoxic nephritis.

Results: All biopsies with CGN had extracellular glomerular MPO deposition that correlated significantly with eGFR and percentage crescent formation. *In vitro,* AZM198 led to a significant reduction in MPO activity, ROS production and released human neutrophil peptide levels and attenuated neutrophil-mediated EC damage. *In vivo*, delayed AZM198 treatment reduced proteinuria, glomerular thrombosis, serum creatinine and glomerular macrophage infiltration (all, $p<0.05$), without increasing adaptive T cell responses.

Conclusion: MPO inhibition reduced AAV patient neutrophil degranulation and attenuated neutrophil-mediated EC damage. In a preclinical model of CGN, delayed MPO inhibition suppressed kidney damage without augmenting adaptive immune responses, suggesting it may be a novel adjunctive therapy in various forms of CGN.

Introduction

Crescentic glomerulonephritis (CGN) describes a severe form of glomerular inflammation that results from various systemic or renal specific diseases and is characterised by glomerular neutrophil and monocyte activation. Myeloperoxidase (MPO), a heme-containing peroxidase mainly active in the phagolysosome, is released following neutrophil and monocyte activation. MPO is activated by hydrogen peroxide (H_2O_2) and catalyses the formation of reactive intermediates such as hypochlorous acid (HOCl) and other reactive oxygen species (ROS) [1]. Although MPO is a key component of innate immune defence [2], MPO deficiency does not appear to lead to a clinical phenotype [3]. However, HOCl and other ROS can lead to host tissue damage by oxidizing/chlorinating a variety of targets, including proteins, lipids and DNA [4].

Neutrophils are also known to release neutrophil extracellular traps (NETs), structures composed of decondensed chromatin decorated with granule-derived antimicrobial peptides and enzymes, such as neutrophil elastase and MPO [5]. Recent data have shown that MPO is necessary for the formation of NETs [6]. Neutrophils from donors who are completely

deficient in MPO fail to form NETs in response to Candida infection [7], and the induction of NETs by Tumour necrosis factor (TNF)-α in healthy subjects requires enzymatically active MPO [8].

NETosis has been linked to a wide array of autoimmune diseases (systemic lupus erythematosus(SLE), ANCA associated vasculitis(AAV), rheumatoid arthritis, cystic fibrosis, and gout) and in recent years NETs have taken centre stage in autoimmunity research as potential links in breaking immune tolerance to innate immune response molecules [9]. NETs are intimately associated with CGN and have been found in the circulation of patients with AAV and SLE [10–12]. The finding that NETs are selectively loaded with antigens recognized by p-ANCAs [13], coupled with extracellular MPO deposition in inflamed glomeruli from patients with AAV[14], provides an explanation for how MPO present on NETs may act as an autoantigen to promote antigen-specific T and B cell reactivity, and glomerular targeting to enhance CGN. A causative role for MPO in mediating glomerular damage is also supported by murine models of CGN in which acute disease is attenuated in MPO-deficient animals, despite augmentation of adaptive (T cell) immunity [15].

Latterly, selective MPO inhibitors have been developed and in experimental models of heart failure [16], pulmonary hypertension [17] and vasculitis[18] they have been shown to ameliorate disease. In the present study we have investigated the role of MPO in mediating glomerular damage and T cell activation using a novel pharmacological MPO inhibitor, AZM198. AZM198 is a recently developed selective MPO inhibitor that effectively inhibits MPO bioactivity and NET formation by human neutrophils. It has been shown to block MPO activity within granules, with higher doses required for inhibition of intragranular MPO when compared with doses needed to fully impede the activity of extracellular MPO[19].

Methods

Patient samples

Blood and tissue samples from patients presenting with AAV and other forms of glomerulonephritis, CKD disease controls, and healthy controls attending the Royal Free Hospital, London, UK were included in this study. All samples and biopsy specimens were obtained following informed consent (National Research Ethics Committee Reference 05/Q0508/6). In all cases the diagnosis of AAV was confirmed by renal biopsy with a positive ANCA result. MPO activity during active AAV and two-month remission was measured in PR3-ANCA positive patient serum enrolled in the RAVE trial [20]. Eighteen renal biopsies were obtained from patients with various forms of CGN (MPO-ANCA (n=5), Proteinase 3(PR3)-ANCA (n=3), ANCA-negative pauci-immune GN (n=2), IgA (n=4) and SLE (n=4)) and stained for CD15 (Biolegend, cat#301902) and MPO (DAKO, A0398). Clinical and laboratory data were collected from hospital records and pathology archives.

Mice

C57BL/6 wild-type (WT), DO11.10 mice and BALB/c mice, were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were female, 10-12 weeks old with an average weight of 25g. All animals were bred at UCL, housed in individually ventilated cages and fed a standard chow diet. Studies adhered to the Animals (Scientific Procedures) Act 1986 (A(SP)A) and UCL Animal Welfare and Ethical Review Body (AWERB) guidelines for animal experimentation.

Immunohistochemistry

Sections (2 μm) of formalin-fixed, paraffin-embedded tissue specimens were mounted on superfrost plus slides (Menzel, Germany), dewaxed, rehydrated, and pre-treated with antigen retrieval solution Tris-EDTA pH 9 in a pressure cooker for 20 min, blocked in 5% casein solution, and probed with antibodies against CD15 (Biolegend, cat#301902) for 2 hours (1:50 dilution) and MPO (DAKO, A0398) overnight at 4 °C. Sections were visualized with a

VECTOR Red alkaline phosphatase substrate kit (Vector, SK-5100) for CD15 and DAB substrate for MPO and counterstained with hematoxylin. MPO deposition was expressed as a percentage of total captured area from ten random high power-fields using Colour Deconvolution, FIJI software (NIH, Bethesda, MD). The macro evaluated area stained correcting for background threshold and expressed results as area fraction percentage of assessed area. Intra-leukocyte MPO was defined being associated with CD15 (CD15+MPO+ cells). Extracellular MPO was measured as MPO+CD15− staining.

Purification of human immunoglobulin

Human anti-PR3 IgG from and healthy control IgG were purified using HiTrap Protein G column (GE Healthcare) chromatography. Each sample was diluted at a ratio of 1:1 in binding buffer (20mM sodium phosphate, pH 7.0). Plasma from anti-PR3 and healthy control were processed on columns and IgG isolated from each sample, eluted by adding 5ml of elution buffer (0.1M glycine-HCl, pH 2.7) and neutralised with alkalising agent (1M Tris-HCl, pH 9.0). Subsequently, endotoxin was depleted (Detoxi-Gel™ AffinityPak™) and protein concentration was determined by spectrophotometry using a NanoDrop 8000 (Thermo Scientific) instrument. ANCA reactivity on isolated neutrophils was assessed using the Dihydrorhodamine (DHR) assay described below (Supplementary Figure 1).

Neutrophil stimulation and inhibition experiments

Peripheral blood anticoagulated with EDTA was mixed with 6% dextran and granulocytes isolated on a Percoll (Sigma) gradient. Human neutrophils were suspended in RPMI medium (2% AB serum (Sigma), 500U/mL penicillin-streptomycin (Gibco Life technologies)). They were plated alone or stimulated with PMA (20 μM) (Sigma) or TNFα (2 ng/mL) (Bio-Rad Laboratories) and endotoxin-depleted ANCA or normal human IgG (both at 0.2 mg/mL) in

the absence or presence of 10 μM AZM198, a concentration that has been reported to inhibit both intragranular and extracellular MPO activity, as well as NETosis [19].The supernatants were collected at 30 minutes and peroxidase activity measured as described below.

ROS production (Dihydrorhodamine (DHR) assay)

DHR 123 is a non-reduced non-fluorescent molecule that in the presence of hydrogen peroxide is converted to rhodamine 123, which fluoresces at a wavelength of approximately 534nm and can therefore be detected in the FITC (FL-1)-light channel on flow cytometry. Neutrophils (2.5 x 10^6 cells/ml) were loaded with 17μg/mL DHR123 (Calbiochem, UK) together with 5μg/mL Cytochalasin B as well as 2mM sodium azide and incubated in the dark for 10 minutes at 37°C. Cells were primed by incubation with 2ng/ml TNFα for 15 minutes and subsequently incubated with PR3-ANCA(200mg/ml) for 45 minutes and subsequently analysed by flow cytometry.

Circulating MPO and Human Neutrophil Peptide (HNP) 1-3 levels, MPO enzymatic activity and ROS production.

Circulating MPO was measured by ELISA (AbCam) in serum samples. In neutrophil supernatants, HNP 1-3 levels were measured by ELISA (Hycult) and peroxidase activity was measured with 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma), as described previously [21]. A standard curve of MPO was made with human MPO (Sigma, #M6908).

NET visualisation and quantification

After the neutrophil supernatants were collected, cells were stained with Sytox Green (0.5 μM) (Invitrogen, cat#S7020) or fixed and stained with MPO (DAKO) and elastase (DAKO, M0752) visualised by anti-rabbit FITC and anti-mouse Alexa fluor 647 IgG respectively and counterstained with DAPI (2 μg/mL). NETs were visualised under a fluorescent microscope. Ten consecutive high-power fields (HPFs) at 20x magnification were captured. For each image, DAPI and Sytox green (Alexa 488) were visualized. Acquired images were automatically analysed by Fiji analysis software by determining the area of extracellular DNA, using a pixel threshold to exclude potential intracellular staining. Extracellular DNA of NETs was quantified as the cumulative area of positive Sytox green. The ratio of Sytox Green stained area: number of nuclei was calculated, representing the NET area index corrected for the number of imaged neutrophils. A higher index ratio indicates a larger NET area present.

Analysis of endothelial cell damage during endothelial cell- neutrophil co-culture

Human umbilical vein endothelial cells (EC), a gift from Dr Xu Shiwen, Centre for Rheumatology, UCL, were cultured on 0.1% gelatine-coated culture plates in basal EC medium containing low serum (2% FCS) and EC growth supplement (PromoCell). BrdU-labelled EC were seeded into 96-well microtiter plates at 10^4 cells/well. Confluent EC were cultured for 18 h in the presence of TNFα (2 ng/well), then rinsed to remove all traces of TNFα and cultured for an additional 18 hours under the following conditions: EC alone; EC + unstimulated PMNs; EC + activated PMN; EC + activated PMN + AZM198; EC + activated PMN + DNAse. The PMNs (2×10^5 cells/well) were primed by TNF (2ng/mL) and stimulated with PR3-ANCA (200ug/mL). DNase (Sigma) was used at 500 U/mL (Sigma) and AZM198 at 10μM. Supernatants were collected for analysis of BrdU-labelled DNA fragments using a cellular DNA fragmentation ELISA (Roche Diagnostics GmbH, Germany)

and von Willebrand Factor release measured by ELISA (RayBiotech). Immunofluorescence staining of the EC monoculture and EC-PMN co-culture was performed using Vascular Endothelial Cadherin (MAB9381, R&D) and MPO(DAKO) counterstained with DAPI.

Nephrotoxic nephritis (NTN)

Thirty two C57BL/6 mice were pre-immunised subcutaneously with sheep IgG (0.2mg) (Sigma-Aldrich, cat# I5131) in Complete Freunds Adjuvant (CFA) (Sigma-Aldrich, cat#F5881) and five days later received intravenous sheep nephrotoxic serum (NTS, 200 μL) mixed 1:1 with Lipopolysaccharide (E.Coli R515, Hycult) diluted in 0.9% NaCl (5μg/mL, final concentration). One day post-immunisation with NTS 16 mice received AZM198 by gavage and the remaining animals were dosed with vehicle (0.5% hydroxypropyl methyl cellulose in water) every 12hr for 7 days. Two different AZM198 dose regimens were used: 133μmol/kg (n=8) or 400μmol/kg (n=8). Both doses are predicted to inhibit at least 80% of the extracellular MPO-activity over 12h, and the high dose is predicted to also inhibit intragranular activity more than 80% during 4 out of 12h [19]. The allocation of animals to groups and the order of dosing were random. The animals were placed in metabolic cages on day 7 for urine collection and were culled on day 8. Proteinuria was quantified using the sulphosalicylic acid assay [22]. Kidneys were collected in buffered formalin and stained with periodic acid Schiff (PAS) and haemotoxylin-eosin (Sigma) or paraformaldehyde-lysine-periodate (PLP) for immunofluorescence and stained for MPO (DAKO), macrophages (using anti-F4/80, Invitrogen cat#14-4801-81), CD4 cells (using rat anti-mouse CD4 clone GK1.5, BD) and sheep IgG (Sigma). Spleens were removed and sieved, following which red cells were lysed for 5 minutes using RBC lysis buffer (#420301) and single cell suspensions were obtained. Splenocytes were cultured in DMEM (10% FCS) with 10 μg/mL sheep globulin for 72 hours, and subsequently stained for expression of CD4 (GK1.5), CD44 (IM7) and CD62L

(MEL-14), and analysed by flow cytometry (FlowJo software). Free plasma AZM198 concentration was measured in all terminal samples.

Pharmakokinetic study for AZM198

Nine C57BL/6 mice received a single dose of 133μmol/kg AZM198 via oral gavage. Free plasma AZM198 was measured at 2 , 12 and 19 hours post dose (n=3 at each tome point) to define the maximal (C_{max}) and trough (C_{min}) levels and to bridge the data to the terminal samples of the NTN study.

Histological scoring

Murine kidney sections were scored for glomerular thrombosis by counting 25 consecutive glomeruli and scoring each glomerulus (number of glomerular quadrants with thrombosis, score 0-4). Glomerular MPO and F4/80 (both FITC) corrected total cell fluorescence (CTCF) and number of CD4 positive cells per glomerulus were counted using Fiji software on ten consecutive HPFs of 25 consecutive glomeruli.

Glomerular neutrophil accumulation

Eight C57BL/6 mice were dosed with either vehicle (n=4) or AZM198 (n=4) twice a day at 133μmol/kg for 48 hours prior to the injection of nephrotoxic serum (NTS) as described above. Twelve hours after the last dosing and 2 hours after NTS injection kidneys were collected and frozen sections stained for Ly6g (ab25377), a murine neutrophil marker.

DO11.10 T cell adoptive transfer model

Lymphocytes were harvested from the lymph nodes of BALB/c DO11 mice, that are transgenic for an MHC class II-restricted T cell receptor (DO11.10) recognizing an

ovalbumin peptide, and stained with KJ126, a monoclonal antibody against the DO11.10 T cell receptor. KJ+CD4+cells were subsequently injected intravenously into six BALB/c mice ($1.2x10^6$ cells/mouse). The following day, the mice were immunised subcutaneously with 50μL ovalbumin peptide (2mg/mL) mixed 1:1 in incomplete Freund's adjuvant on each side of the chest wall. Subsequently, the mice were dosed twice daily by oral gavage with vehicle or AZM198 (400umol/kg) for 7 days, following which they were sacrificed, and their draining lymph nodes were harvested. Cells were stained for DO11.10, CD4, and CD44 and analysed using flow cytometry (BD) and FlowJo software.

Circulating antibodies

Circulating serum levels of mouse anti-sheep globulin IgG subclasses, IgG1, IgG2b and IgG3 were assessed by ELISA (Invitrogen) according to the manufacturer's protocol.

Statistical Analysis

The results from animal and patient studies are expressed as medians (interquartile range). All statistics were performed using GraphPad prism 8.0 (GraphPad Software, San Diego California, USA). Non-parametric tests of significance were applied. For comparing two groups, Mann-Whitney U test was used, for groups of three or more, one-way analysis of variance (ANOVA) was carried out using a nonparametric Kruskal-Wallis test and Dunn's multiple comparison. Two-way ANOVA was used to analyse differences among groups from more than one experiment. Wilcoxon signed-rank test was used to compare non-parametric paired data. Correlations were assessed using the non-parametric Spearman rank correlation analysis. A significant value was defined $p<0.05$ with 95% confidence.

Results

Serum MPO levels are elevated in patients with active ANCA-associated crescentic glomerulonephritis

Serum MPO levels were measured in patients with active ANCA-associated glomerulonephritis (AAGN) and AAGN disease in remission, as well as in patients with renovascular disease and in healthy controls. Increased MPO levels were found in patients with active PR3-AAGN 482 (330.0-1397) ng/mL compared with active MPO-AAGN 204.3(42.6-396.7) ng/mL ($p=0.04$) and healthy controls, 96.2 (53.5- 176.3) ng/mL ($p < 0.001$) (Figure 1A). However, we found that MPO-ANCA-containing serum inhibited MPO detection in the immunoassay by up to 40% (Supplementary Figure 2), suggesting that the MPO-concentration in MPO-AAGN samples is potentially underestimated. Therefore, we only included patients with PR3-ANCA AAGN when we analysed differences between MPO levels in active AAGN and disease remission (Figure 1B).

We found that median MPO levels in PR3-ANCA patients with active disease from the RAVE trial were significantly reduced when measured at two-months following treatment (active AAGN 262.9 (211.5-355.2) ng/mL *vs* remission 177.5 (128.5-275.2) ng/mL, $p= 0.002$)

Extracellular glomerular MPO deposition is seen in various forms of crescentic glomerulonephritis

We stained for MPO and the neutrophil marker CD15 by immunohistochemistry using biopsies from patients with various forms of crescentic glomerulonephritis (CGN) due to MPO-ANCA (n=5), PR3-ANCA (n=3), ANCA negative pauci-immune GN (n=2), crescentic IgA (n=4) and SLE (n=4) (Figure 2).

Total (leukocyte and non-leukocyte associated) whole kidney MPO deposition significantly correlated with disease severity as defined by estimated Glomerular Filtration Rate (eGFR) ($r = -0.68$, $p<0.001$, Figure 3A) and proteinuria ($r=0.51$, $p=0.02$ Supplementary Figure 3C) as

well as with histological disease severity defined by percentage of glomeruli with active cellular crescents (r= 0.58, p=0.01) (Figure 3B) and chronic damage expressed as percentage of interstitial fibrosis and tubular atrophy (IFTA) on the renal biopsy, (r = 0.66, p= 0.001, Supplementary Figure 3A). Glomerular MPO deposition correlated with eGFR (r=-0.45, p=0.03, Figure 3C) as well as percentage of active crescents (r=0.42, p=0.04, Figure 3D) and IFTA (r= 0.53, p=0.01, Supplementary Figure 3B). Dual staining allowed us to detect the presence of extracellular glomerular MPO, defined as MPO staining (brown) that was not co-localized with CD15 (red). Extracellular MPO was found close to intra-glomerular CD15+ cells, but was also deposited independently along the glomerular capillaries (Figure 2 A-D, yellow arrows) and on tubular epithelial cells. When we analysed extracellular (non-leukocyte associated) glomerular MPO deposition, we found that it correlated significantly with eGFR (r= -0.58, p=0.03, Figure 3E) and crescent formation (r=0.63, p=0.02, Figure 3F, Supplementary Table 1).

MPO inhibition inactivates enzymatically active MPO and reduces neutrophil degranulation, ROS production and NET formation *in vitro*

We measured the enzymatic activity of MPO, ROS production, human neutrophil peptide (HNP) 1-3 levels, and visualised NET formation in neutrophils from patients with AAV and from healthy controls in the absence and presence of AZM198. HNP 1-3 are found in the azulophilic granules of human neutrophils and were chosen as a neutrophil degranulation marker independent of myeloperoxidase. In this way, we aimed to assess whether MPO activity could potentially drive further neutrophil degranulation, ROS production and NETosis.

Neutrophils were stimulated with TNFα/PR3-ANCA or PMA, a synthetic activator of protein kinase C, in the presence or absence of AZM198. Supernatants were collected after 30 minutes and peroxidase activity per gram of myeloperoxidase was measured. AZM198

attenuated enzymatically active myeloperoxidase activity from PMA-stimulated neutrophils (PMA 37.4(9-175.6) U/g *vs* AZM198 12.93(1.9-152.8) U/g, p<0.001; Supplementary Figure 4A).

In TNFα primed and PR3-ANCA stimulated neutrophils from patients and healthy controls, we found that addition of AZM198 led to a significant reduction in ROS production (Figure 4A) and HNP1-3 levels (Figure 4B) (MFI Rhodamine 123: PR3-ANCA 10675(4481-13196) AU, *vs* unstimulated neutrophils 1749 (515-3808) AU (p=0.004) and AZM198 2123 (643-6275) AU(p=0.04), HNP 1-3 levels: PR3-ANCA 135(116-217) pg/mL *vs* unstimulated neutrophils 106 (97-160)pg/mL, p=0.003 and AZM198 127(104-189)pg/mL, p=0.03). TNFα/PR3-ANCA stimulation triggered NET formation, characterized by Sytox green binding of extracellular DNA structures in cells from active AAV patients (open squares) and healthy controls (open circles) while AZM198 reduced NETosis (Figure 4C) (NET area index PR3-ANCA 6.3 (5.6-7.2)AU *vs* unstimulated neutrophils 2.7 (0.9-3.3) AU and AZM198 3.3(1.9-4.2) AU, p= 0.01). Representative images of NETosis induced by TNFα/PR3-ANCA and PMA stimulation in the presence and absence of AZM198 are shown in Figures 4D-F and Supplementary Figures 4B-C.

In summary, these data demonstrate that AZM198 inactivates enzymatically active peroxidase activity *in vitro*, while reducing neutrophil degranulation, ROS production and NETosis.

Endothelial cell/ PR3-ANCA-stimulated neutrophil co-culture results in EC damage that is reduced by MPO inhibition

Neutrophil interaction with activated EC is required for cell transmigration, but recent reports have shown that activated EC can also induce NET formation that can promote EC death [23–25].

To quantify EC damage after co-culture with healthy control neutrophils (n=5), we used TNFα-primed BrdU-labelled EC and measured release of BrdU-labelled DNA fragments and von Willebrand Factor (vWF) into the co-culture supernatants. There was measurable EC death when co-cultured with TNFα/PR3-ANCA-stimulated neutrophils (BRDU 0.44±0.04AU), which was reduced with the inclusion of DNase (0.36±0.05, p=0.03) as well as AZM198 (0.34±0.04AU, p=0.01, Figure 5A). However, we did not detect a significant reduction in supernatant vWF levels in the presence of AZM198 (PR3-ANCA 259±40 ng/mL *vs* AZM198 197±28 ng/mL, p=0.06, Figure 5B). EC morphology was visibly disrupted during the EC-PMN 18hr co-culture when PMN were stimulated, but this was attenuated in the presence of AZM198 Figure 5C-F. Taken together these data demonstrate that a neutrophil-NET-mediated mechanism of EC injury can be significantly reduced by using MPO inhibition.

MPO inhibition attenuates glomerular inflammation in the NTN model

During the initial, heterologous phase of NTN, injury is mediated by infiltrating neutrophils whose accumulation in glomeruli peaks 2 h after the administration of nephrotoxic serum (NTS) [26]. The autologous phase of the disease develops within the subsequent 7 days and is initiated by CD4 T cells against the immunizing antigen and macrophages. To confirm that MPO inhibition would also be protective *in vivo*, we assessed the effect of AZM198 in a 7-day and 2-hour mouse NTN model.

One day following NTS injection and following the time of early neutrophil entry into the kidney mice received vehicle (n=16) or AZM198, at 133μmol/kg (n=8) or 400μmol/kg (n=8) by gavage twice a day for 7 days. Two animals in the vehicle group (on day 6 and 7) and one in the 400μmol/kg AZM198 group (day 2) died, while two vehicle animals were anuric. MPO inhibition using AZM198 at either dose led to a reduction in renal injury compared with the

vehicle group. In particular, median (IQR) glomerular thrombosis score in the 133 μmol/kg group was 0 (0-0.1) and in the 400 μmol/kg group 0.8 (0.5-1.3) and *vs* vehicle 2.6 (1.0-3.3) ($p<0.001$ and $p=0.24$, respectively, Figure 6C-E). There was also a reduction in proteinuria in both AZM198-treated groups compared with vehicle (133 μmol/kg 1.3 (1.0-1.9) mg/24hrs and 400μmol/kg 1.0 (0.1-1.2) mg/24hrs *vs* vehicle 3.5 (1.0-7.1) mg/24hrs, $p=0.22$ and $p=0.02$, respectively) (Figure 6A). Plasma creatinine was also lower in both AZM198 groups compared with vehicle (plasma creatinine 133 μmol/kg 12.5 (9.0-14.8) μmol/L and 400 μmol/kg 6.0 (1.2-8.0) μmol/L and vs vehicle 16.1 (8.4-52.8), $p=0.26$ and $p=0.04$, respectively, Figure 6B). Immunofluorescence staining for F4/80, a murine macrophage marker, MPO and murine CD4 showed a significant reduction in macrophage and CD4 positive T cell infiltration and glomerular MPO deposition in the kidneys of the animals that were treated with AZM198 133μmol/kg compared with vehicle controls. (F4/80 CTCF (Corrected Total Cell Fluorescence) in AZM198 41.1 (24.0-42.3) AU *vs* vehicle 68.2 (58.3-75.6) AU $p=0.01$, number of T cells per glomerulus AZM198 0 (0-0.4) *vs* vehicle 1 (0 -2.1) and MPO CTCF in AZM198 45.9 (40.6-51.6) AU *vs* vehicle 67.7 (61.0-102.0) AU, $p=0.01$) (Figure 7A-G and Supplementary Figure 5C-D).

To assess the effect of AZM198 on glomerular neutrophil accumulation, the initial phase of NTN, we dosed mice with either vehicle (n=4) or AZM198 (n=4) twice a day at 133μmol/kg for 48 hours. On day 3, ten hours after the last AZM198 dosing, NTS was administered and kidneys were collected 2 hours later, for assessment of glomerular neutrophil infiltration (Fig 7H and Supplementary Figure 5A-B). We found a non-significant reduction in glomerular neutrophil infiltration in those treated with AZM198 (Ly6g CTRF AZM198 26.5(23-70.7) AU *vs* vehicle 71.0(45.3-152.8) AU, $p=0.11$). Taken together the *in vivo* experiments suggest the delayed effect of AZM198 may be on neutrophil activation and the downstream effects of

extracellular MPO mediated inflammation, rather than initial neutrophil glomerular recruitment.

Effect of MPO inhibition on adaptive immunity

Previous reports have suggested that in murine models of CGN, acute glomerular disease is attenuated in MPO-deficient animals, but there is augmentation of adaptive T cell immunity[15,27]. To investigate if this also occurs after pharmacological inhibition of MPO, we determined the effect of AZM198 on T- and B-cell responses in context of two different models. We investigated the effect on humoral and cellular immunity in the Th1 and Th17-mediated and immune complex-driven NTN model described above, and the effect on antigen-specific T cell responses in an adoptive transfer model of T cell receptor transgenic T cells recognizing an ovalbumin peptide, i.e. the DO11.10 mice.

To assess the effect of MPO inhibition on humoral immunity in the NTN mice, circulating serum titres of mouse anti-sheep immunoglobulin subclasses were measured by ELISA. There was no significant increase in the concentration of IgG1, IgG2b and IgG3 subclasses in the AZM198-treated animals compared with vehicle controls (IgG1: 354.5 (33.6-465.2) μg/mL *vs* vehicle 347.3 (212.5-994.5) μg/mL, p=0.38 and IgG2b 191.8 (73.9 -305.3)μg/mL *vs* vehicle 163.0 (30.9-297.7) μg/mL p=0.51, IgG3 8.4 (5.1-50.6) μg/mL *vs* vehicle 16.0 (3.4-46.6) μg/mL p>0.99 (Figure 8B-D).

T cells play a fundamental role in inducing damage in NTN and we have previously shown that AZM198 led to a significant reduction in glomerular T cell infiltration. To assess further the effect of MPO inhibition on cellular immunity in the NTN mice, we used the fact that naive T cells exhibit high levels of CD62L and low expression of CD44, whereas activated and memory T cells have the reciprocal expression of these markers. CD4+ splenocytes from the NTN mice that received vehicle or AZM198 133 μmol/kg, were isolated and re-stimulated with sheep globulin *in vitro* for 72 hours, after which expression of CD44 and

CD62L were assessed by flow cytometry (Figure 8A). There was no difference in the median frequency of $CD44^{high}CD62L^{low}$ T cells amongst the vehicle and treatment group, suggesting that AZM198 attenuated glomerular inflammation without an increase in adaptive T cell responses (median $CD44^{high}CD62L^{low}$ % of CD4+cells: AZM198 49.6(42.9-55.9)% *vs* vehicle 47.6(38.6-58.2)%, (p=0.78)).

Finally, we tested the effect of pharmacological MPO inhibition on antigen-specific T cell responses, using adoptive transfer of DO11.10 lymphocytes into OVA immunised mice dosed with vehicle or AZM198 (Figure 8E-F). Seven days post-immunization, the frequency of activated CD4 cells and activated OVA-specific T cells in draining lymph nodes were determined by staining for CD4, CD44 and the DO11.10 T cell receptor. Treatment with AZM198 did not result in higher frequency of activated total (CD4, CD44) or OVA-specific (CD44, KJ) T cells. Rather, AZM198-treated mice tended to have reduced frequencies of these T cell populations. ($CD44^{high}$% on CD4+ cells: AZM198 12.6% (11.8-13.8) *vs* vehicle 19.35% (16.5-19.9) p=0.10, $CD44^{high}$KJ+% on CD4+ cells (AZM198 1.9(1.64-3.14) *vs* vehicle 4.1(3.9-5.0), p=0.05). These results suggest that pharmacological MPO inhibition, unlike genetic MPO-deficiency, does not augment antigen-specific T cell responses in the draining lymph nodes.

Discussion

We have shown that MPO can be detected both intra- and extracellularly in inflamed glomeruli, in biopsies from patients with the most common form of CGN in adults, ANCA-Associated Vasculitis, as well as in ANCA negative disease, crescentic IgA nephropathy and Lupus Nephritis. Total, neutrophil-associated and extracellular MPO deposition in the whole kidney as well as total glomerular MPO deposition correlated with clinical and histological disease severity in the initial renal biopsy. Extracellular glomerular MPO deposition alone

correlated with eGFR and crescent formation, suggesting that extracellular MPO could be an important driver mediating renal inflammation. Our data on the distribution of extracellular and intracellular MPO within kidneys of AAV patients confirm and extend previous observations [10,14,28,29]. Additionally, we measured circulating MPO levels and showed increased MPO levels in AAV patients with active disease compared with disease in remission and healthy controls, confirming it as a modifiable marker of disease activity.

We also showed that MPO inhibition with AZM198 inactivated enzymatically active MPO and reduced neutrophil degranulation, ROS production and NET formation in cytokine- and ANCA-stimulated neutrophils from patients and healthy controls.

Recent studies have provided evidence that support the central role for NETs in the pathophysiology of different forms of crescentic glomerulonephritis. Impaired NET degradation in patients with SLE has been associated with higher dsDNA titres and frequency of flares [30]. Cocaine and levimasole have been shown to induce NET formation, suggesting that NETs can act as potential sources of autoantigen in cocaine-associated autoimmunity that clinically resembles the systemic vasculitis in AAV[31]. Furthermore, in MPO-ANCA positive CGN a reduced degradation of NETs has been observed, implicating NETs as a means of breaking tolerance to ANCA autoantigens [32]. The latter was further corroborated in animal studies where presentation of extracellular DNA derived from NETotic neutrophils to myeloid dendritic cells led to MPO-ANCA and PR3-ANCA production, with subsequent vasculitis-like renal lesions in C57BL/6 mice [33]. In addition, augmented NET formation following infection in dectin-/- mice leads to a vasculitic phenotype, which can be attenuated by inhibiting NET generation [34].

There has been recent insight regarding the processes regulating neutrophil degranulation and NETosis, but the exact molecular mechanisms remain to be elucidated. Upon neutrophil stimulation, the oxidative burst generates H_2O_2 that triggers the activation of MPO which

subsequently catalyses the formation of reactive oxygen intermediates and ROS. ROS stimulate MPO to trigger the activation and translocation of neutrophil elastase from azurophilic granules to the nucleus, where neutrophil elastase proteolytically processes histones to disrupt chromatin packaging. Subsequently, MPO binds chromatin and synergizes with neutrophil elastase in chromatin decondensation and NET formation[35].

One of the hallmarks of crescentic glomerulonephritis is endothelial injury and rupture of the glomerular capillary loops due to aberrant leucocyte activation. Cytokine activated EC produce IL-8, which has a significant role in promoting neutrophil recruitment and migration, [36,37]. Recent reports have shown that activated EC not only interact with neutrophils during transmigration, but may also induce NETosis, leading to EC death [23,38]. Both ROS and serine proteases such as PR3 and elastase released during neutrophil degranulation and presented on NETs have been previously proposed as initiators of endothelial cell damage in vasculitic lesions *in vivo* [39,40]. We aimed to replicate this neutrophil- EC interaction *in vitro* and found that TNFα/PR3-ANCA stimulated neutrophils led to EC injury that was attenuated by enzymatic NET degradation using DNAse [23,38], as well as pharmacological inhibition of MPO with AZM198.

We used the accelerated nephrotoxic nephritis model of crescentic glomerulonephritis, which is dependent on adaptive and innate immunity and representative clinically of a rapidly progressive glomerulonephritis due to immune complex disease. We performed therapeutic treatment with administration of two different doses of the inhibitor one day following injection of the nephrotoxic serum. Despite the initiation of treatment after the glomerular neutrophil influx, AZM198 at both doses attenuated glomerular inflammation clinically and histologically. When we assessed the effect of AZM198 on neutrophil influx we found no significant difference in neutrophil influx at an early time point. Acknowledging the small sample size of animals tested in this experiment (n=4 in each group) we may have missed a

biological effect on neutrophil recruitment which has been shown in previous reports[15]. Alternatively, our results may be explained by an effect of the delayed MPO inhibition on neutrophil activation/degranulation and inhibition of the biological effects of enzymatically active MPO in addition to any effect on neutrophil recruitment.

AZM198 is a membrane-permeable compound that acts as a suicide substrate for MPO and has been shown to inhibit enzymatically active extracellular MPO, while at higher concentrations it also inhibits intragranular MPO together with NET formation[19,41]. The rationale for the dose setting in the current experiments was to yield exposures that would mainly inhibit extracellular (133 µmol/kg) or extracellular as well as intragranular MPO (400 µmol/kg). A satellite group of mice were dosed with 133 µmol/kg AZM198 and sampled at 2, 12 and 19h to define the maximal (C_{max}) and trough (C_{min}) levels and to bridge the data to the terminal samples of the efficacy study. The observed pharmacokinetic responses are shown in Supplementary Figure 6, which also shows the concentration of AZM198 predicted to inhibit 80% of extracellular MPO and 80% of glutathione sulfonamide formation/ peroxidase activity in human whole blood (the latter corresponding to the intragranular MPO potency). The reported IC_{50} of AZM198 is 0.015 mM. Therapeutic AZM198 levels were achieved throughout the study. Our PK data suggest that the peak plasma concentrations our MPOi achieved in the current experiments were in the range that are found to inhibit intragranular MPO, even using the lower dose of MPOi.However, the limited benefit of 400 over 133 µmol/kg might argue that some beneficial effect of AZM198 on glomerular inflammation in this model may also be due to the inhibition of extracellular MPO activity.

The NTN model dissociates the effect of the AZM198 from the induction and perpetuation of autoreactivity. As there is no autoimmunity in this model and the treatment is delayed, we can reasonably assume that this is testing MPO inhibition largely for its effects in the kidney

and not on the induction or maintenance of immunity to sheep Ig. Our data confirm and extend previous observations made by Zheng *et al*.(2015), which showed that prophylactic MPO inhibition attenuated disease in a mild, non-crescentic murine anti-GBM model and a model of lung vasculitis[18].

Importantly, our methodology differs in that we used an accelerated severe nephrotoxic nephritis model with features of glomerular thrombosis and administered the MPO inhibitor a day after the injection of the nephrotoxic serum, reflecting human disease and clinical presentations to a greater extent. Moreover, we demonstrate for the first time that there is no impact on adaptive T cell responses, a critical requirement for translation of such therapy to the clinic in the setting of autoimmune crescentic glomerulonephritidies. Previous data using MPO-deficient mice have suggested a short-term improvement in glomerulonephritis following immunisation with sheep anti-mouse glomerular basement membrane globulin, but increased adaptive T cell responses [15,27].

As the one-year survival of patients with AAV has improved from 20% to over 80% over the last 5 decades, current clinical needs have focused on the long-term outcome [42,43]. There is still significant morbidity in vasculitis patients associated with current treatments, and there are also forms of CGN such as IgA nephropathy for which we have limited effective treatments. Glucocorticoid therapy is associated with significant long-term morbidity and recent trials in CGN have been designed to address the need for steroid minimisation (PEXIVAS, CLEAR, ADVOCATE and our recent steroid free maintenance AAV cohort study) [44–47] .

In conclusion, we have demonstrated that therapeutic MPO inhibition reduced degranulation and NET formation in neutrophils from patients with AAV, and attenuated kidney damage in preclinical models of CGN without augmenting adaptive immune responses, suggesting that MPO inhibition may be an effective and novel adjunctive therapy in various forms of CGN.

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Figure 1

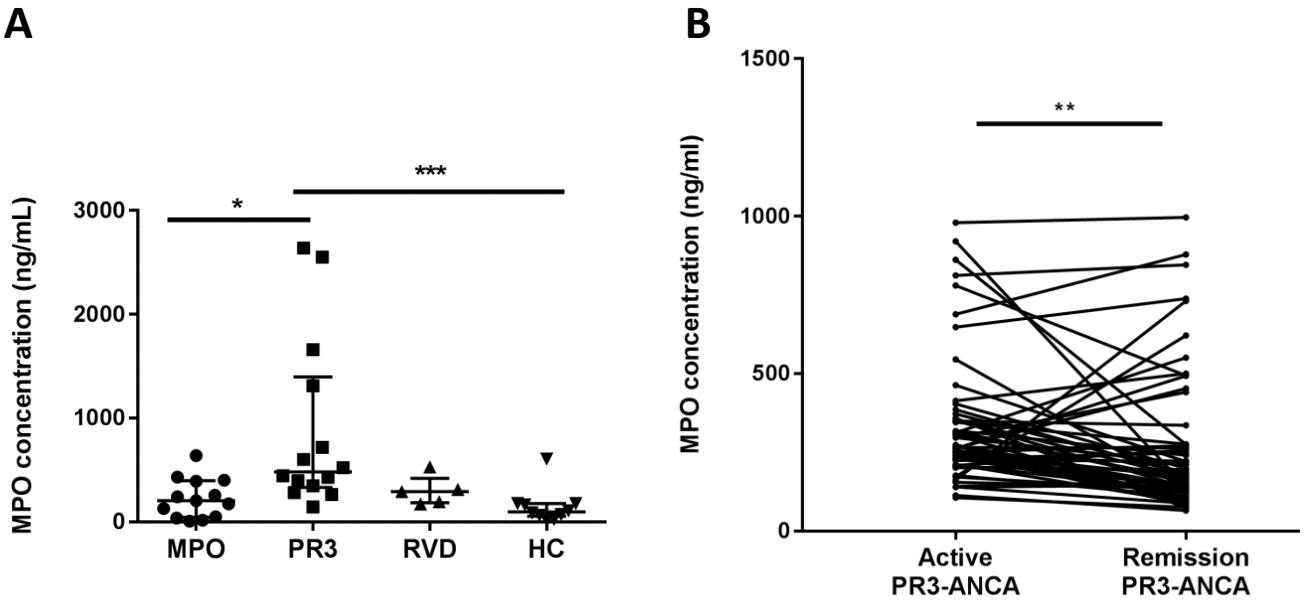
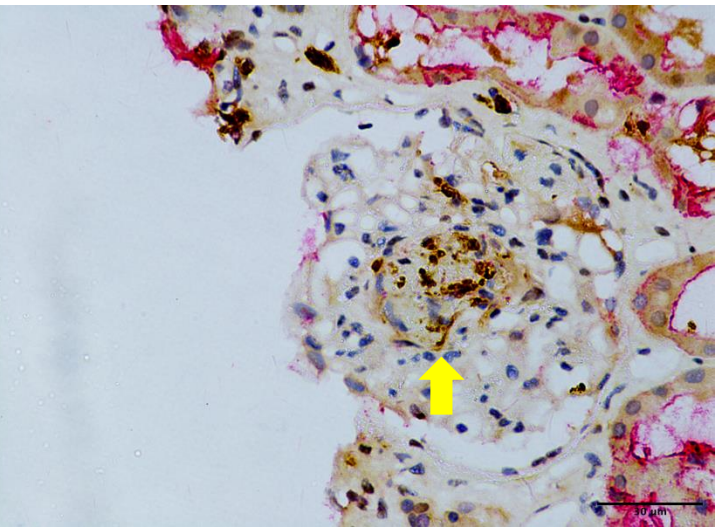
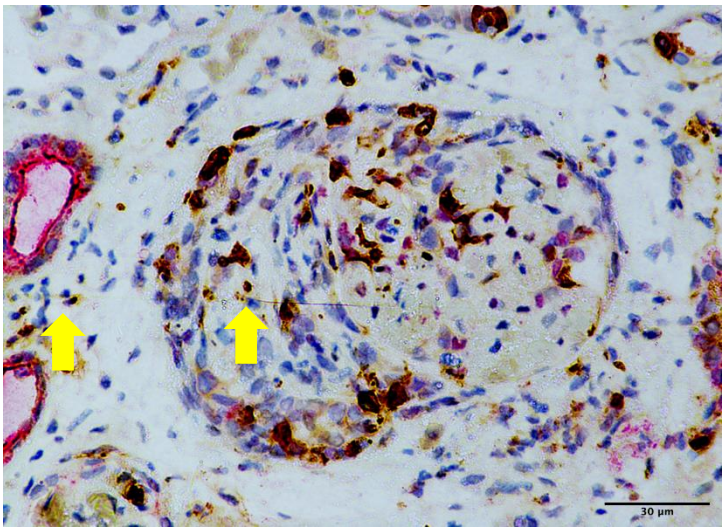

A
B
MPO concentration (ng/mL)
3000
2000
1000
0
*
***
MPO
PR3
RVD
HC
MPO concentration (ng/ml)
1500
1000
500
0
**
Active
PR3-ANCA
Remission
PR3-ANCA

Figure 2

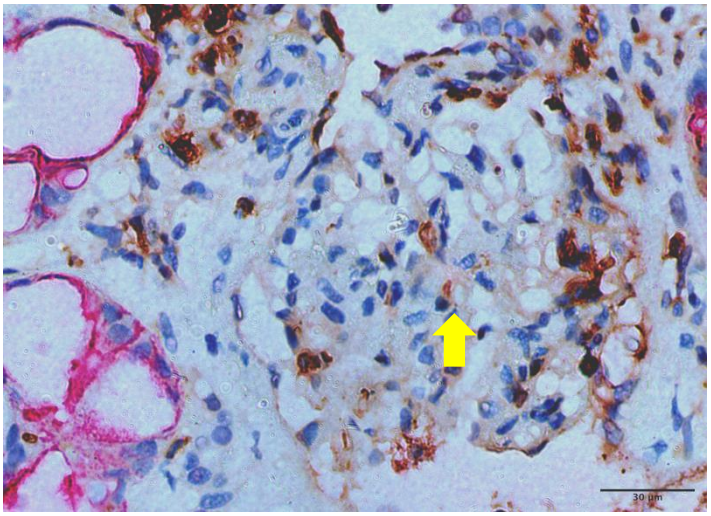
A



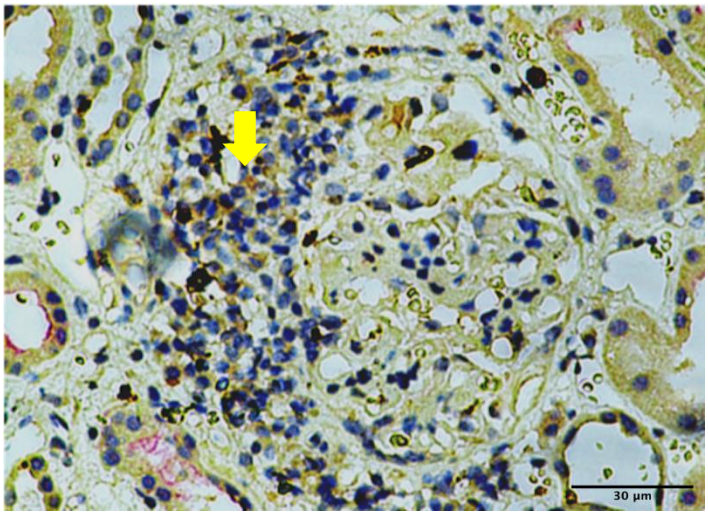
B



C



D



E

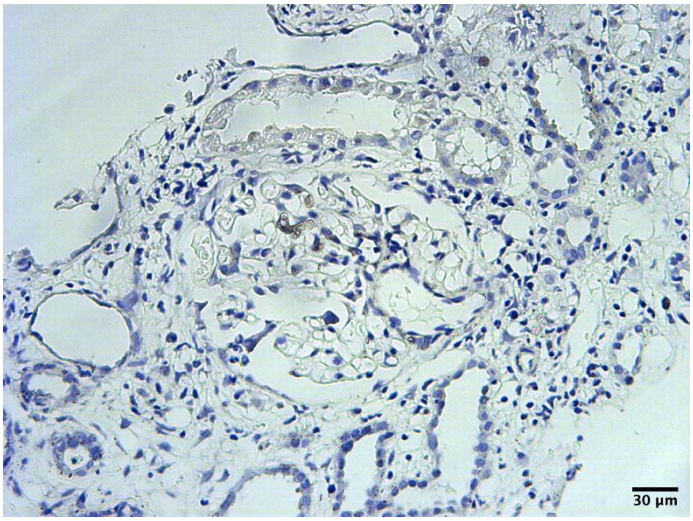


Figure 3

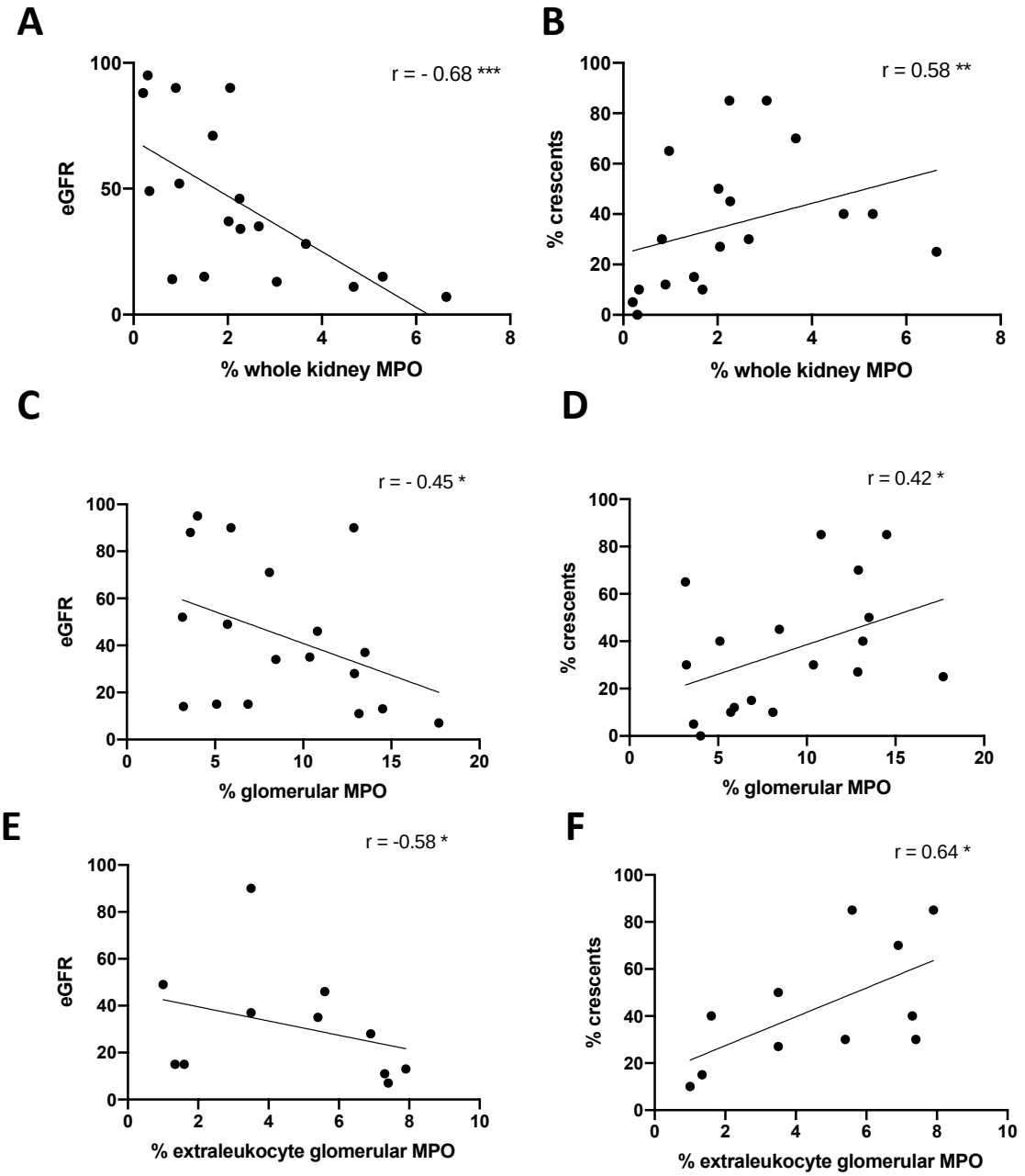
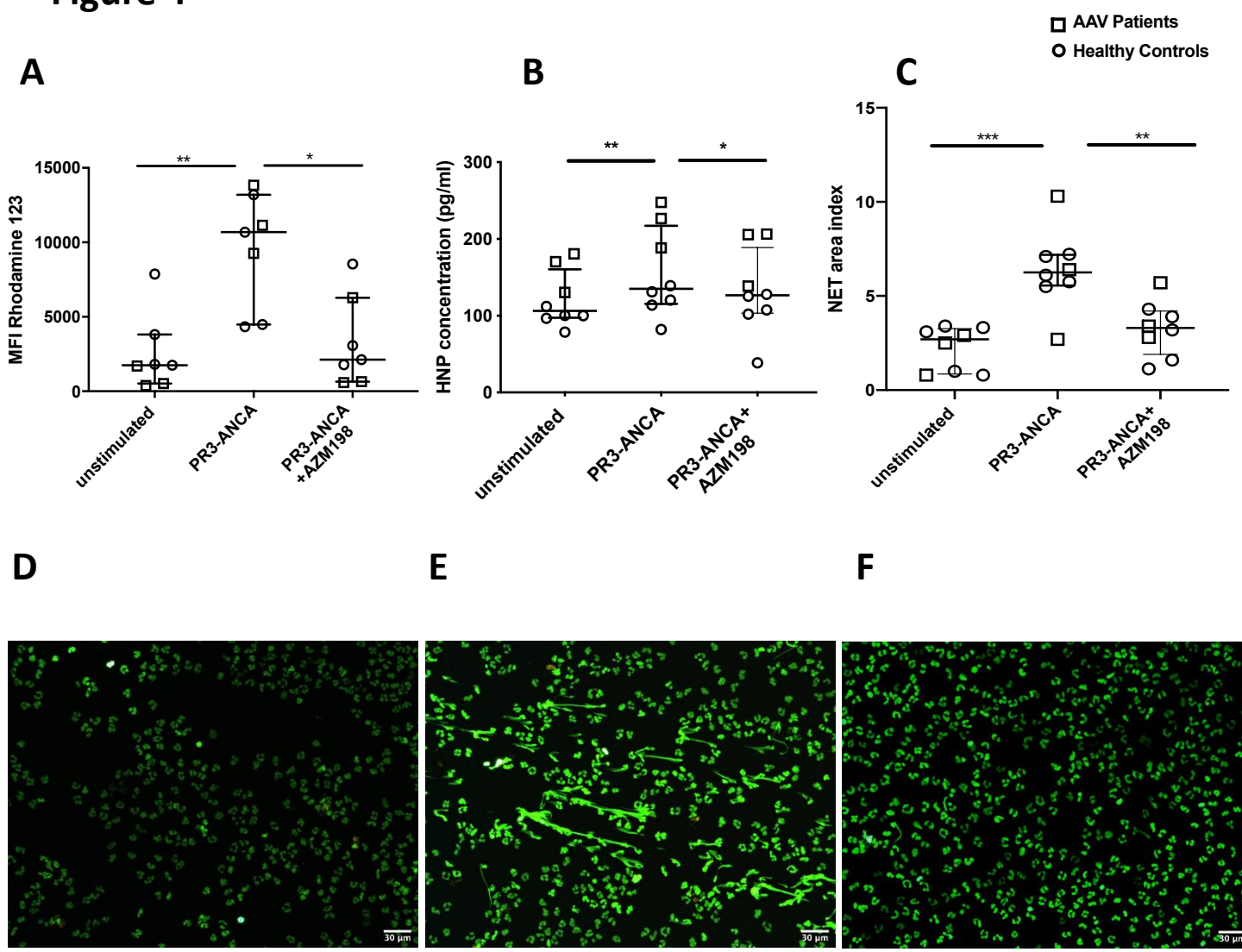

A
r = - 0.68 ***
eGFR
% whole kidney MPO
B
r = 0.58 **
% crescents
% whole kidney MPO
C
r = - 0.45 *
eGFR
% glomerular MPO
D
r = 0.42 *
% crescents
% glomerular MPO
E
r = -0.58 *
eGFR
% extraleukocyte glomerular MPO
F
r = 0.64 *
% crescents
% extraleukocyte glomerular MPO

Figure 4


A
B
C
AAV Patients
Healthy Controls
MFI Rhodamine 123
HNP concentration (pg/ml)
NET area index
unstimulated
PR3-ANCA
PR3-ANCA +AZM198
PR3-ANCA+ AZM198
**
*
***

D
E
F
30 µm

Figure 5

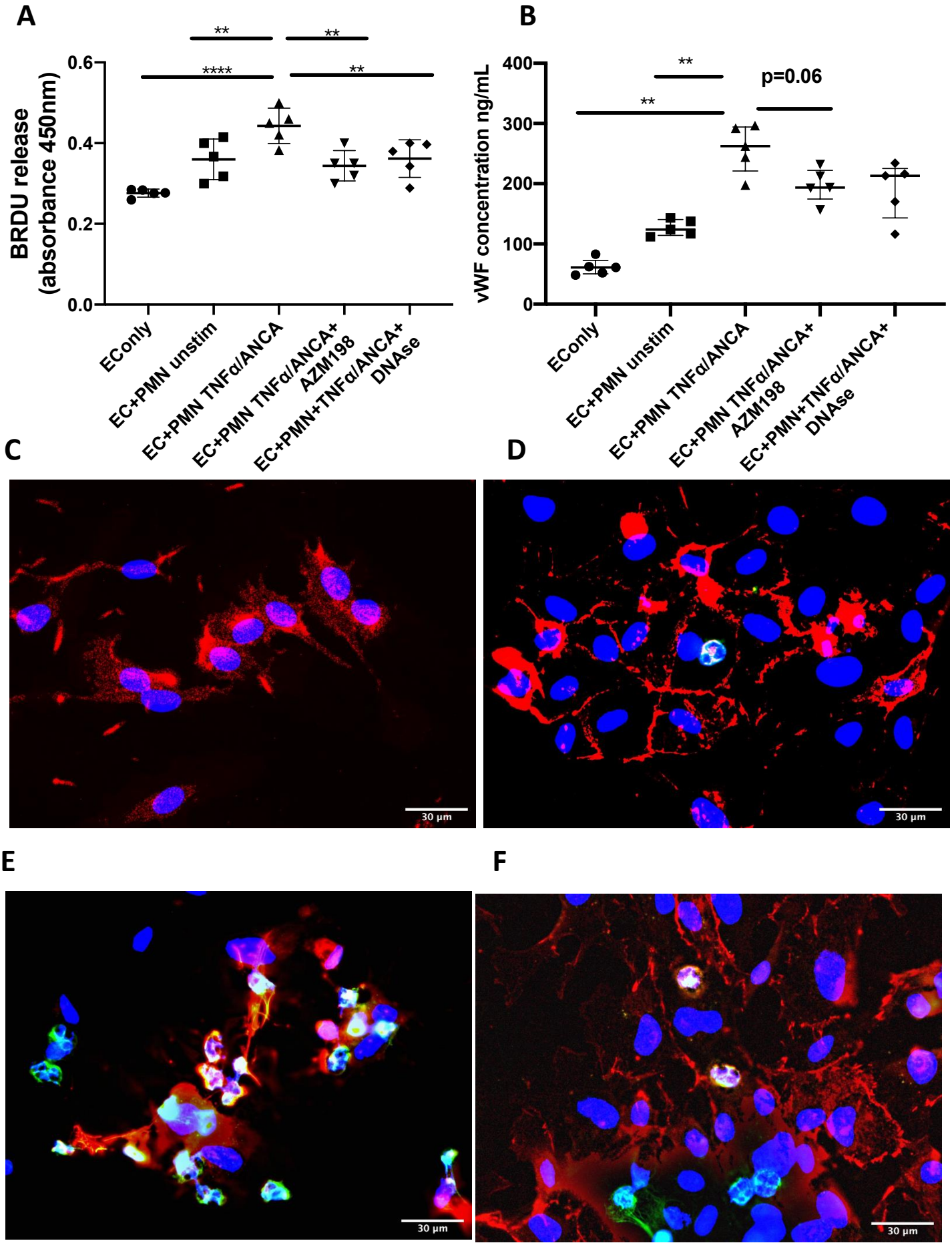
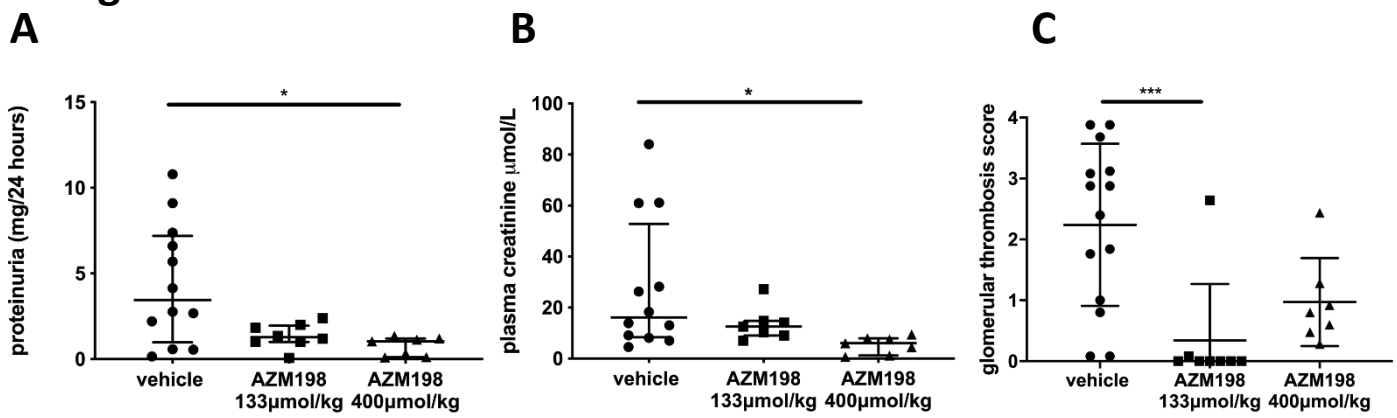
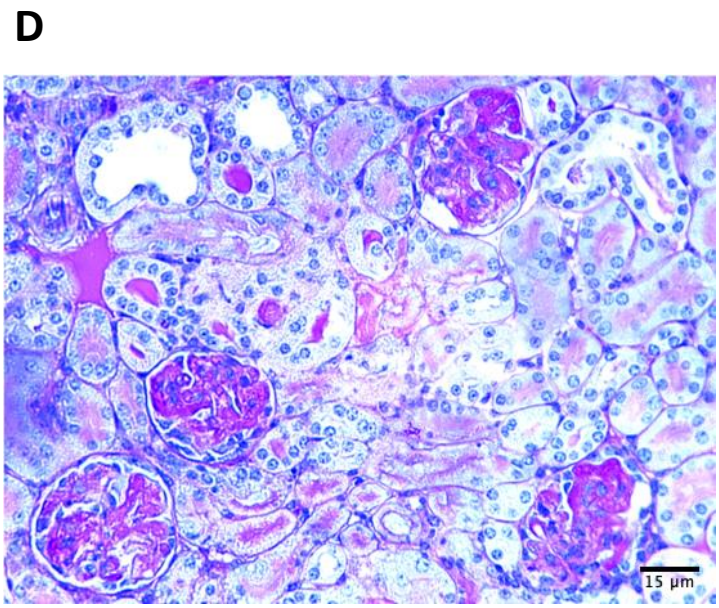

A
B
BRDU release (absorbance 450nm)
vWF concentration ng/mL
p=0.06
EConly
EC+PMN unstim
EC+PMN TNFα/ANCA
EC+PMN TNFα/ANCA+ AZM198
EC+PMN+TNFα/ANCA+ DNAse
C
D
E
F
30 µm

Figure 6


A
B
C
proteinuria (mg/24 hours)
plasma creatinine μmol/L
glomerular thrombosis score
*
*
***
vehicle
AZM198 133μmol/kg
AZM198 400μmol/kg


D
15 μm

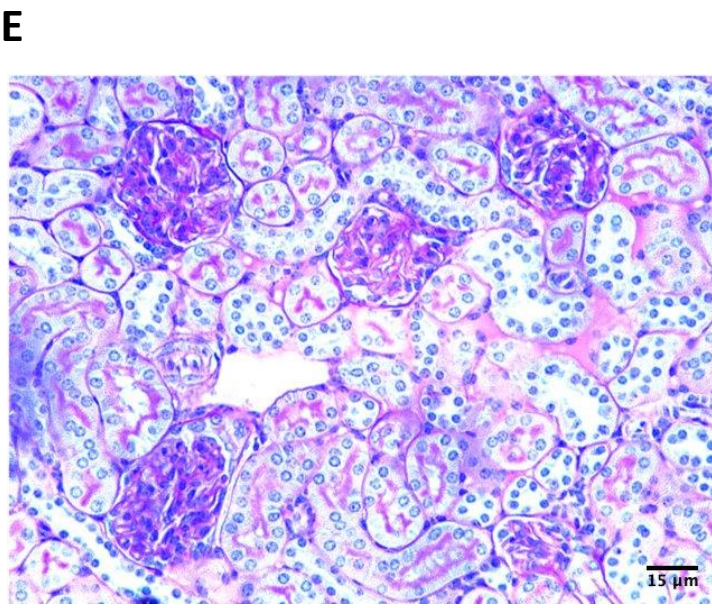

E
15 μm

Figure 7

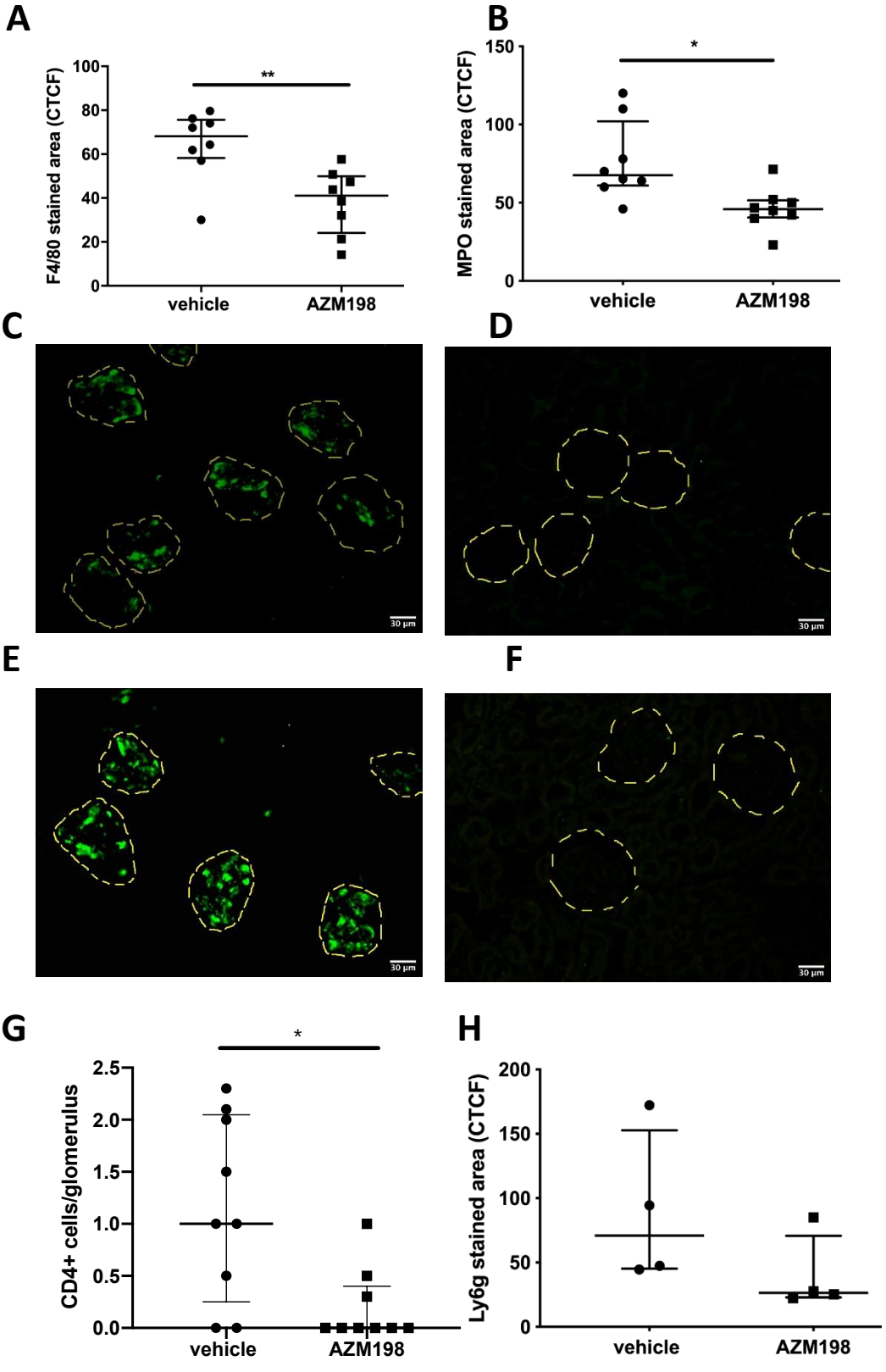
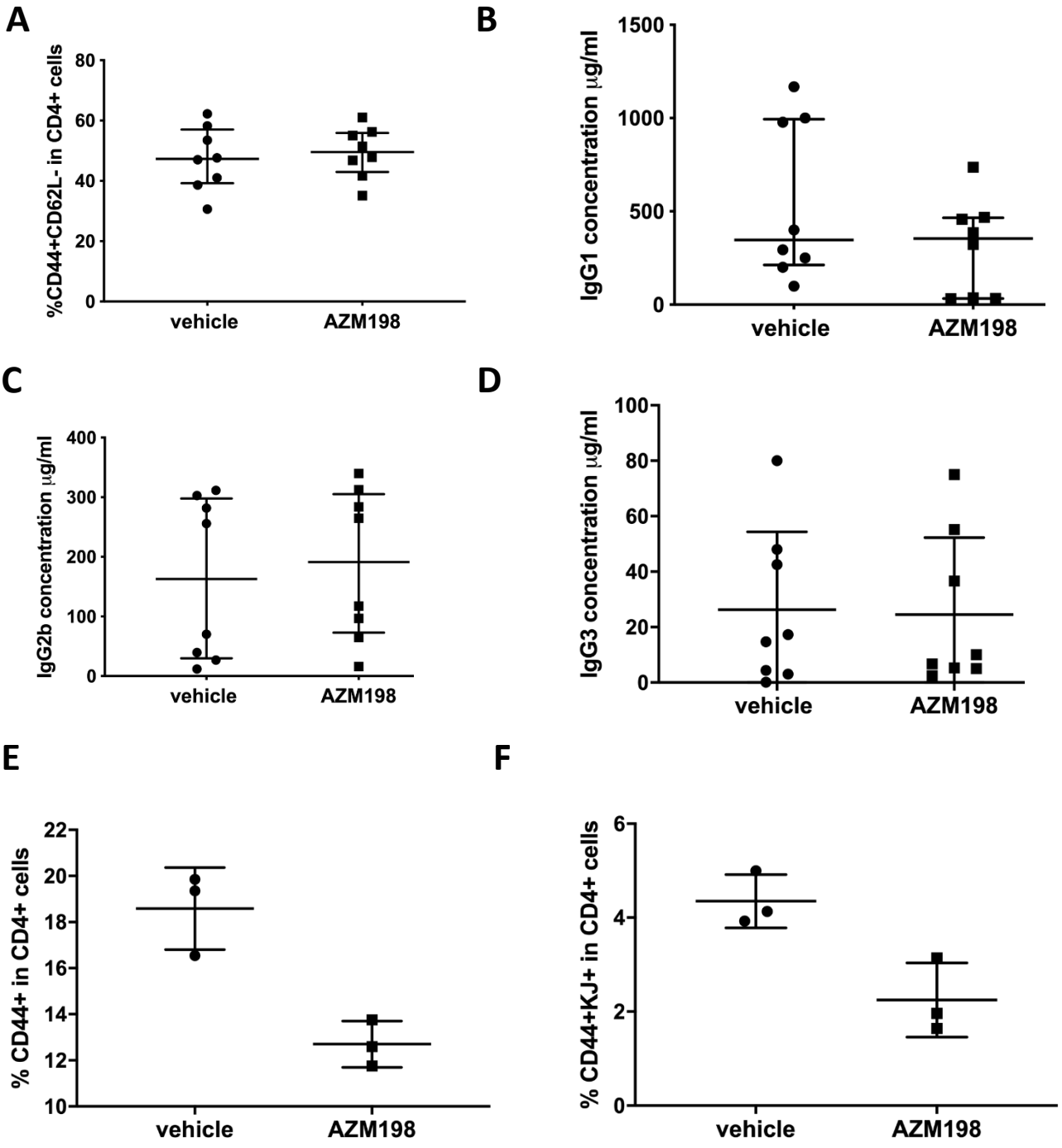
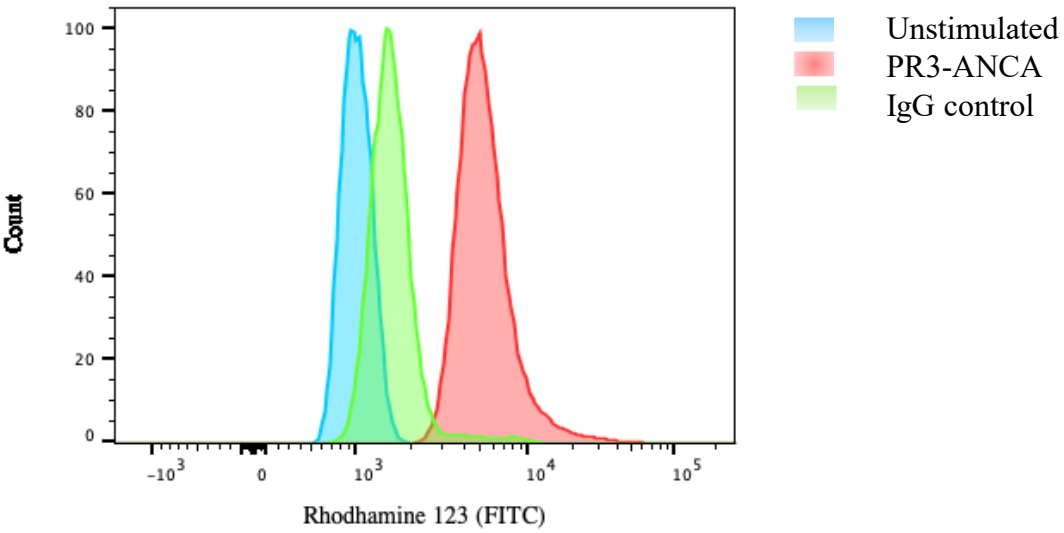

A
F4/80 stained area (CTCF)
100
80
60
40
20
0
**
vehicle
AZM198
B
MPO stained area (CTCF)
150
100
50
0
*
vehicle
AZM198
C
30 µm
D
30 µm
E
30 µm
F
30 µm
G
CD4+ cells/glomerulus
2.5
2.0
1.5
1.0
0.5
0.0
*
vehicle
AZM198
H
Ly6g stained area (CTCF)
200
150
100
50
0
vehicle
AZM198

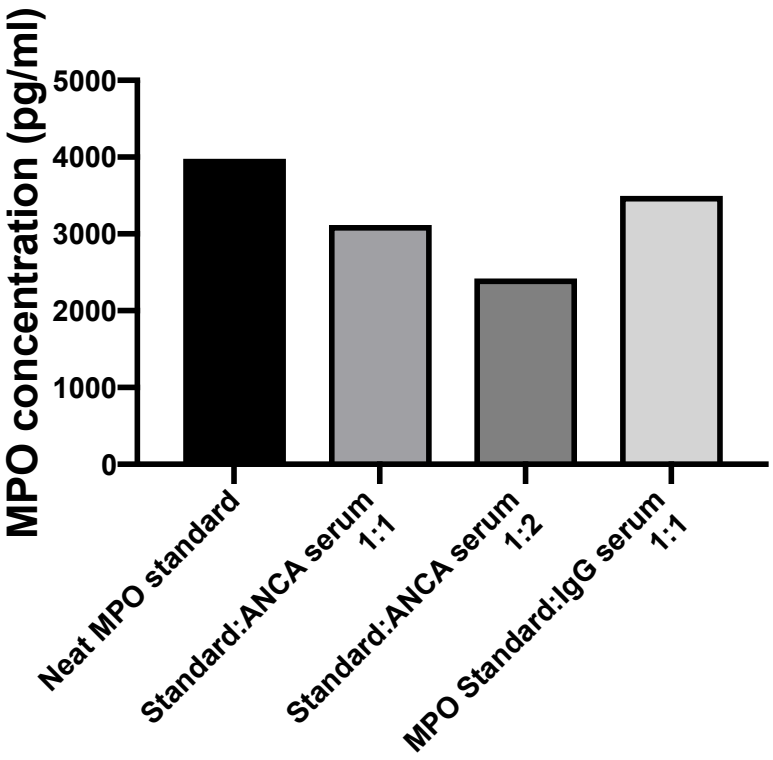
Figure 8


A
%CD44+CD62L- in CD4+ cells
80
60
40
20
0
vehicle
AZM198
B
IgG1 concentration µg/ml
1500
1000
500
0
vehicle
AZM198
C
IgG2b concentration µg/ml
400
300
200
100
0
vehicle
AZM198
D
IgG3 concentration µg/ml
100
80
60
40
20
0
vehicle
AZM198
E
% CD44+ in CD4+ cells
22
20
18
16
14
12
10
vehicle
AZM198
F
% CD44+KJ+ in CD4+ cells
6
4
2
0
vehicle
AZM198

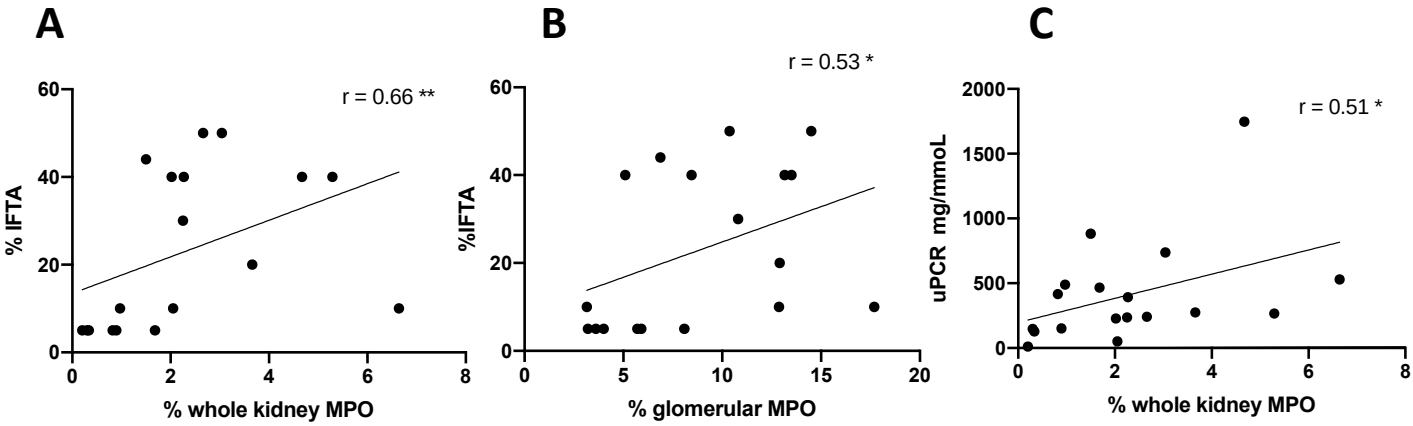
Supplementary Figure 1



Supplementary Figure 2

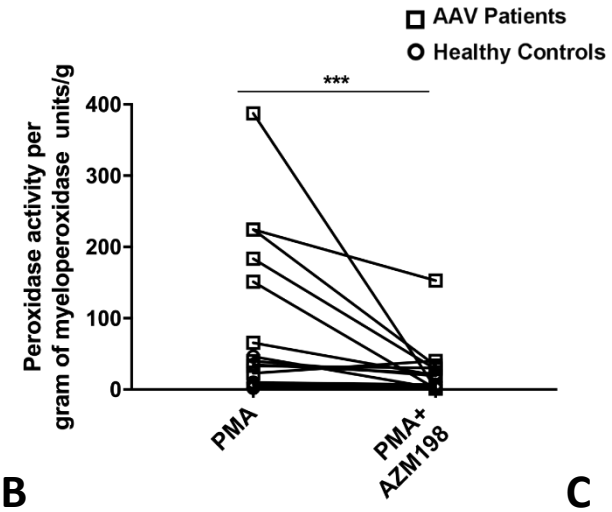


Supplementary Figure 3

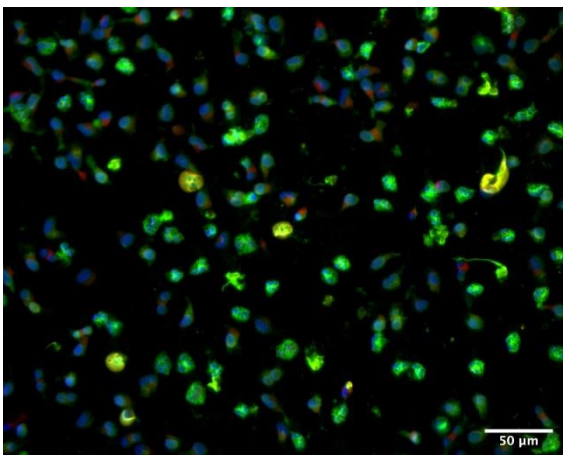


Supplementary Figure 4

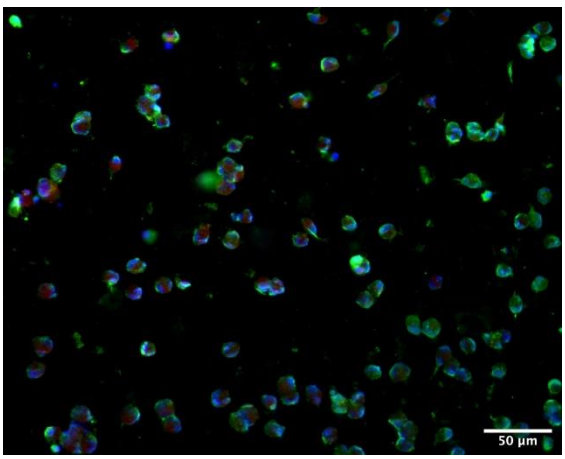
A



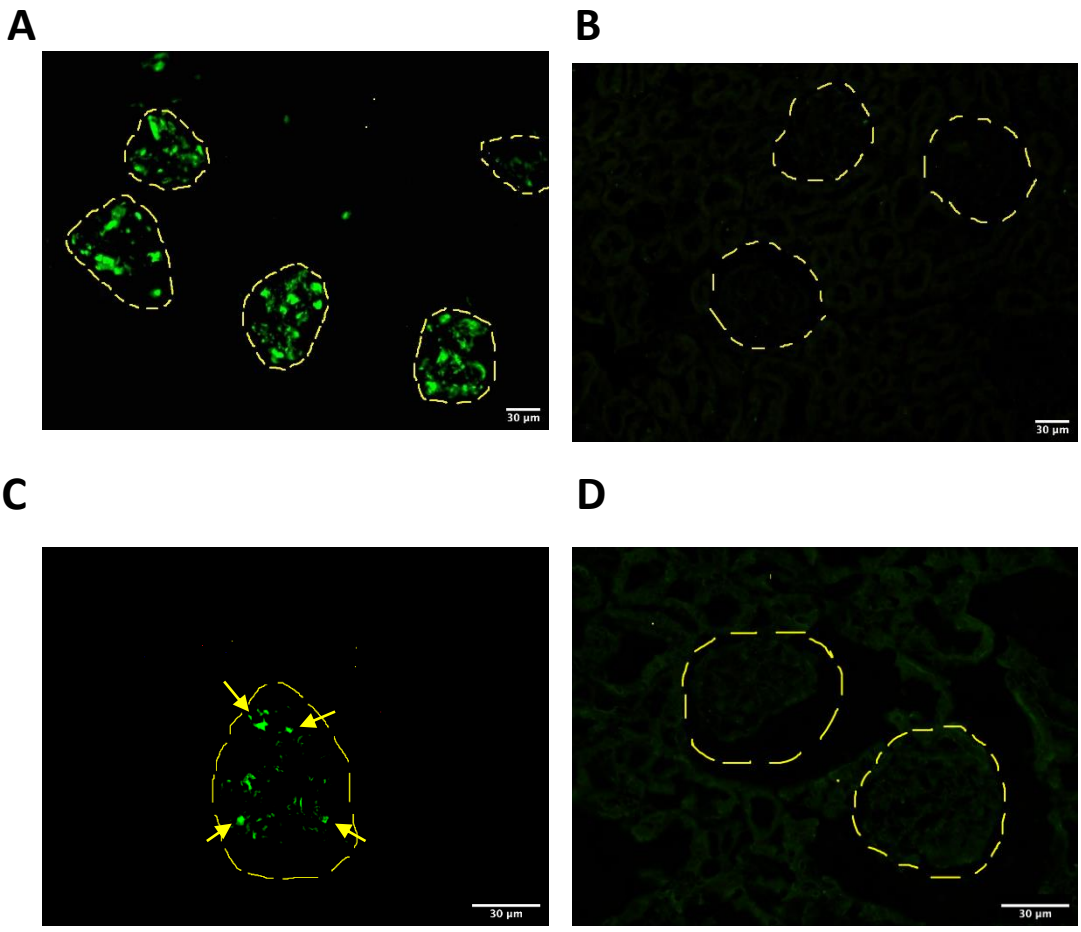
B



C



Supplementary Figure 5



Supplementary Figure 6