Anti-neutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions in vivo

Running head: ANCA enhance microvascular responses in vivo

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**Abstract**

Systemic small vessel vasculitis is associated with anti-neutrophil cytoplasm antibodies (ANCA). Whilst there is mounting *in vitro* evidence to suggest that ANCA are capable of enhancing leukocyte-endothelial interactions, no *in vivo* evidence for this has been provided. In this study a novel rat model of ANCA-associated experimental autoimmune vasculitis (EAV), induced by immunisation with human MPO, was used to analyse directly the potential effect of ANCA on leukocyte-venular wall interactions *in vivo* as observed by intravital microscopy. These rats developed anti-MPO antibodies directed against rat leukocytes, showed pathological evidence of small vessel vasculitis, and had enhanced leukocyte adhesion and transmigration in response to the chemokine Groα (CXCL1). Passive transfer of immunoglobulin from rats with EAV to naïve rats conferred enhanced adhesion and transmigration responses in the recipients. Furthermore, rats with EAV and recipients of ANCA-positive immunoglobulin developed extensive microvascular injury, as manifested by mesenteric haemorrhage, in response to CXCL1. This study provides the first direct *in vivo* evidence for the ability of ANCA to enhance leukocyte-endothelial interactions and cause microvascular haemorrhage, thereby providing a mechanism by which ANCA could exert pathogenic effects in systemic vasculitis.
**Introduction**

Systemic small vessel vasculitis (SVV) results in rapidly progressive glomerulonephritis and lung haemorrhage in humans, and is usually fatal if untreated\(^1\). It is characterised by microvascular inflammation and necrosis in a variety of organs. The two organs that are most extensively injured in this manner are the kidney, through the development of pauci-immune crescentic glomerulonephritis, and the lung, with consequent alveolar haemorrhage. The association between ANCA and SVV, initially described in the 1980s\(^2\), has attracted considerable interest over the past decade. These autoantibodies are principally directed against myeloperoxidase (MPO) and proteinase-3\(^3,4\). At first, ANCA were regarded only as clinical markers of disease activity, but it is now apparent that they have direct biological effects on neutrophils\(^5,6\) and monocytes\(^7\). The binding of ANCA to antigen expressed on the leukocyte cell surface following cytokine priming is followed by the activation of an array of intracellular signalling pathways\(^8\), with resultant degranulation and dysregulated apoptosis\(^9\).

Granulocyte infiltration and fibrinoid necrosis of the vessel wall are the pathological hallmarks of SVV. For this reason, investigative efforts have focussed on the influence that ANCA have on the interaction between leukocytes and the vascular endothelium, specifically with reference to the inflammatory cascade of leukocyte rolling, adhesion and transmigration. In this context, *in vitro* studies using flow chamber models have shown that ANCA can cause rolling neutrophils to arrest on platelet monolayers\(^10\), and promote firm adhesion and migration of rolling neutrophils on endothelial cells\(^11,12\). Despite growing *in vitro* evidence implicating ANCA as an inducer of leukocyte-
endothelial cell interactions, there has been no evidence to date demonstrating such an
effect of these antibodies *in vivo*. The ability of ANCA to induce systemic small vessel
vasculitis and crescentic glomerulonephritis has, however, recently been demonstrated by
transfer of anti-MPO antibodies in a mouse model of vasculitis\textsuperscript{13}. Crescent formation in
these animals occurred secondary to fibrinoid necrosis of the glomerular tuft, which was
induced by necrosis and occlusion of glomerular capillaries.

To investigate the mechanisms involved in the initiation of inflammatory microvascular
injury, we have used a rat model of ANCA-associated SVV (AASV) that mimics the
human condition, recently developed in our laboratory\textsuperscript{14}. In this model, experimental
autoimmune vasculitis (EAV), Wistar-Kyoto (WKY) rats, immunised with purified
human myeloperoxidase (hMPO) in CFA, develop high titres of ANCA, accompanied by
pauci-immune crescentic glomerulonephritis and lung haemorrhage. As the aim of our
study was to investigate the hypothesis that ANCA are capable of promoting leukocyte-
endothelial interactions *in vivo*, leukocyte responses within the mesenteric vascular bed
of immunised rats were directly investigated using intravital microscopy. The findings
demonstrate increased leukocyte firm adhesion and transmigration, as induced by locally
administered CXCL-1 (a rat homologue of IL-8), in rats immunised with hMPO, and in
naïve rats following passive transfer of ANCA from rats with EAV. This was
accompanied by microvascular injury, as shown by mesenteric haemorrhage in response
to CXCL-1. Collectively, these results provide the first direct *in vivo* evidence that
ANCA can cause enhanced leukocyte-endothelial interactions and support the concept
that ANCA are pathogenic.
Methods

Animals

WKY / NCrlBR rats were purchased from Charles River (Margate, UK). All animal studies were performed according to the directives of the United Kingdom Home Office Animals (Scientific Procedures) Act, UK (1986).

Immunisation protocol and characterisation of EAV phenotype

Rats (130-180g) were immunised i.m. with hMPO (400, 800 and 1600µg/kg; Calbiochem, Merck biosciences, Nottingham, UK, n=13,11 and 4 respectively) or HSA (Sigma-Aldridge, Gillingham, UK, n=19) in an equal volume of CFA. Haematuria was assessed with dipstick (Bayer multistix, Berkshire, UK) and albuminuria with ELISA (Nephrat ELISA kit, Exocell, Philadelphia, PA, USA). Anti-hMPO antibody levels in serum were measured by ELISA. Briefly, 96-well plates were coated overnight with hMPO (2µg/mL) in carbonate buffer. Wells were then incubated with dilutions of serum samples in triplicate for 60 minutes at 37°C, washed and incubated with anti-rat IgG alkaline phosphatase conjugate (Sigma) for 45 minutes at 37°C. Binding was detected with p-NPP (Sigma) and read at 405nm.

Following intravital microscopy, rats were sacrificed and organs were fixed in formalin (for histological analysis), snap-frozen (for immunofluorescence staining) and fixed in glutaraldehyde for analysis by electron microscopy. Glomerular crescent percentage was quantified blindly in 100 random glomeruli on each periodic acid Schiff stained section. Direct immunofluorescence staining for glomerular IgG deposition was performed on
3μm frozen sections after fixation in acetone for 10 minutes. Following washing in PBS, non-specific binding was blocked by incubation with 20% normal rabbit serum and sections were then incubated with FITC-conjugated anti-rat IgG (Sigma) for 60 minutes. To control for the thickness of the section, images were captured digitally using a laser scanning confocal microscope and Pascal LSM software (LSM5 Pascal, Zeiss, Germany).

**Ig preparation**

Ig from pooled rat sera of all actively immunised animals was prepared by 45% ammonium sulphate precipitation. Final Ig preparations had endotoxin levels of <5 EU/mL, as measured by Limulus Amebocyte Lysate testing (Bio-Whittaker, Walkersville, USA). All Ig preparations were ultracentrifuged (10^5 g x 30 minutes) and the presence of anti-hMPO antibodies in preparations from rats with EAV was confirmed using ELISA.

**Intravital microscopy**

Intravital microscopy on rat mesentery was performed as described previously. Briefly, control (n=18) or EAV (n=20) rats were anaesthetised with i.v. sodium pentobarbitone, without the operator’s knowledge of the immunisation status of the rat, and maintained at 37°C on a heated microscope stage. Following midline abdominal incision, the mesentery adjoining the terminal ileum was carefully arranged over a glass window on the stage and superfused with Tyrode’s balanced salt solution. Baseline quantification of leukocyte rolling, firm adhesion and transmigration was performed using a fixed stage upright microscope with water immersion objectives (Axioscope, Zeiss). To investigate the effect
of recombinant rat CXCL1 (Peprotech, London, UK), the chemokine was continuously applied topically to the mesentery in the superfusion buffer (final concentration $3 \times 10^{-9}$M) and leukocyte responses were quantified at regular intervals. In experiments investigating passive transfer of ANCA, non-immunised rats were injected in a blinded fashion with ANCA-containing (n=9) or control (n=8) Ig (20mg/kg i.v.).

In animals that developed extensive haemorrhage, each vessel segment (all of which had been chosen randomly before the addition of a stimulus) was assessed for the presence or absence of haemorrhage at each time point. The number of haemorrhagic venular segments was then expressed as a percentage of the total number of vessel segments observed. This quantification procedure was, however, insensitive for accurate recording of responses in rats with minor degrees of haemorrhage, as seen in the passive transfer experiments. In these, haemorrhage at the vessel segments chosen at the beginning of the experiment was infrequent. In these cases haemorrhage was quantified using a global visual-analogue score, defined in dose-finding experiments as 0=no haemorrhage, 1=a single focus, 2=2-5 foci, 3=6-10 foci, 4=>10 foci with macroscopic petechiae visible and 5=widespread haemorrhage with extensive microvascular occlusion. The experiments also typically involved measurement of peripheral differential leukocyte counts, blood pressure and RBC velocity, as previously detailed\textsuperscript{16}.

**Assessment of anti-hMPO cross-reactivity with neutrophils from WKY rats**

Binding of anti-hMPO antibodies to WKY rat neutrophils was demonstrated by indirect immunofluorescence staining, by flow cytometry and by Western blotting of leukocyte
lysates. With respect to fluorescence staining, neutrophil-rich leukocyte suspensions were prepared from healthy human volunteers and non-immunised WKY rats by discontinuous density centrifugation. Smears were air-dried and fixed in 95% ethanol before incubation with serum (1:20 in PBS) from EAV (n=13) or control (n=10) rats, or plasma (1:20 in PBS) from anti-MPO antibody positive patients (microscopic polyangiitis, n=5) or anti-MPO negative controls (IgA nephropathy, n=2; antibody mediated transplant rejection, n=1). In addition, staining following serial dilution of selected sera to 1:320 was performed. Binding of antibodies was detected using appropriate Alexa Fluor 568-conjugated anti-rat/human IgG antibodies (Molecular probes, Oregon, USA). Samples were observed using a laser scanning confocal microscope (LSM5 Pascal, Zeiss, Germany) with the aid of Pascal LSM software. Fluorescence intensity was quantified using Image-Pro plus software (v3.0, Media Cybernetics, Maryland, USA).

For flow cytometry, mixed leukocyte preparations from WKY rats and healthy human volunteers were prepared by sedimentation of red cells using 2.5% Dextran T-500 (Amersham biosciences, Chalfont, UK) with permeabilisation of cell samples with Saponin (0.1% in PBS). Surface and intracellular antigen expression was detected with Ig preparations from EAV or control rats (100µg/mL in PBS / 20% rabbit serum) and a FITC-conjugated rabbit anti-rat IgG (Sigma). In selected experiments granulocytes were distinguished by double staining with PE-conjugated HIS48 IgM (Santa Cruz biotechnology, California, USA). The samples were acquired on a FACSCalibur flow cytometer (Becton Dickinson). Binding of EAV Ig relative to control Ig was expressed as mean RFI.
For Western blot analysis, mixed leukocytes were prepared as for flow cytometry, lysed and run on SDS-PAGE. Briefly, after lysing residual RBCs with ice-cold NH₄Cl lysis solution, the leukocyte pellet was suspended in lysis buffer (1% Triton x100, 10mM Tris, 5mM EDTA, 50mM NaCl, 30mM Na₄O₇P₂, 50mM NaF, 100mM Na₃VO₄, pH 7.6) containing protease inhibitors (Sigma) and incubated on ice for 20 minutes. The samples were centrifuged at 14,000g for 20 minutes at 4°C and non-denaturing SDS-PAGE was performed on the lysate supernatant with a Phast-gel system using 7.5% agarose gels (Amersham Pharmacia Biotech, Uppsala, Sweden). The purified hMPO (50µg/mL) used to induce EAV and lysates of human PMN’s purified over percoll were used as positive controls. Samples were transferred to nitrocellulose membranes and incubated with primary antibodies (Ig preparations from rats with EAV and control rats), followed by incubation with appropriate anti-rat IgG alkaline phosphatase conjugates, with all incubations being for 60 minutes. Binding was detected using BCIP/NBT alkaline phosphatase substrate (Sigma).

**Statistical analysis**

Normally distributed data are expressed as the mean ± SEM and non-normal data are expressed as median and interquartile range (IQR). Baseline variables were compared using Student’s t-test, and urinary albumin excretion rate and haemorrhage at 90 minutes was analysed with Mann-Whitney test. Intravital microscopy adhesion and transmigration data were analysed using 2-way ANOVA, considering time and treatment group as...
independent variables. Values at individual time points were compared using Bonferroni
post-hoc tests.
Results

Immunisation of WKY rats with hMPO induces anti-hMPO antibodies that bind to WKY rat neutrophils

To assess the effect of circulating ANCA on microvascular leukocyte-endothelial responses, WKY rats were immunised with hMPO (EAV group) or HSA (control group) in CFA. As there were no differences in any of the outcome variables studied between the 3 hMPO dosing groups used (400, 800 and 1600µg/kg), for all analyses the groups receiving different doses of hMPO were considered together. Since binding of anti-hMPO antibodies to rat neutrophils was critical to our in vivo investigations, initial experiments evaluated this using a number of techniques.

Using ELISA, all rats immunised with hMPO had titres of anti-hMPO antibodies in excess of 1:1000, and Ig preparations from rats with EAV had detectable anti-hMPO activity at concentrations above 0.5µg/mL. Rats immunised with HSA, or Ig prepared from these rats, had no detectable anti-hMPO activity. As EAV was induced with human antigen, it was essential to assess the ability of ANCA from rats with EAV to bind to rat MPO (and rat neutrophils) as a prerequisite for demonstrating any potential biological effect. This was established by immunofluorescence staining, by flow cytometry, and by Western blot analysis of neutrophil lysates.

Observation of ethanol-fixed rat and human neutrophils by confocal microscopy (Figure 1, Panel 1), after indirect staining with various sera, indicated that anti-hMPO antibody
positive sera from EAV rats bound to WKY neutrophils in a cytoplasmic pattern, whereas they bound to human neutrophils in a typical perinuclear pattern. The pattern of binding of EAV rat sera to human or rat neutrophils was similar to the pattern produced by human MPO-ANCA positive sera from patients with vasculitis on the same substrates. Fluorescence intensity on WKY cells remained above that obtained with anti-hMPO negative serum up to a dilution of 1:320. The intensity of binding of rat anti-hMPO positive serum to rat neutrophils was less than that observed with human neutrophils, but consistently greater than that produced by anti-hMPO antibody negative sera. These observations are in agreement with Image-Pro quantification of fluorescence intensity (Figure 1, Panel 2) and with flow cytometry results confirming the binding of Ig from EAV rats (but not control Ig) to rat and human granulocytes (Figure 1, Panel 3) with RFI values of 2.0 and 6.9, respectively (n=4 separate experiments). Considering the cell population staining positive for the rat granulocyte marker, HIS48, the percentage of cells staining positive for FITC increased from 14%, when incubated with control Ig, to 72% when incubated with EAV Ig.

The binding of Ig preparations from hMPO immunised and control rats to WKY and human neutrophils was also investigated by Western blotting (Figure 1, Panel 4). Immunoglobulin preparations from rats with EAV bound to several bands in the purified hMPO and human leukocyte lanes, the strongest being at 126kDa and 74kDa, and to a single 120kDa band in the WKY rat leukocyte lane. Under denaturing conditions, this band was at 59kDa, suggesting that it is the heavy chain of MPO. No binding was observed to either human or WKY lysates using Ig from control rats. Collectively, these
findings confirm that the polyclonal antibodies that develop in hMPO immunised EAV rats bind to antigen in WKY rat neutrophils.

**Immunisation of WKY rats with hMPO induces pauci-immune crescentic glomerulonephritis and pulmonary haemorrhage**

Gross examination of lungs and kidney revealed petechiae over the lung surface, and occasional petechiae on the renal surface in rats with EAV, but not in control rats. Prominent macroscopic lung haemorrhage was evident in 41% of rats immunised with hMPO. In all of these, there was histological evidence of lung vasculitis, with perivascular leukocyte cuffing, occasional vascular occlusion and alveolar haemorrhage (Figure 2). In 79% of hMPO-immunised animals, but none of the control animals, focal proliferative glomerulonephritis with fibrinoid necrosis was evident (Figure 2). In 78% of these (61% of all hMPO-immunised rats), crescent formation was present (Figure 2). This was focal, with adjacent areas of normal renal tissue, and often associated with foci of tubulo-interstitial nephritis. Immunofluorescence examination of renal sections revealed occasional scanty deposits of IgG. The amount of deposits was above the background level seen with sections from animals immunised with CFA alone, but much less than that seen with rats with experimental autoimmune glomerulonephritis, a model of anti-glomerular basement membrane disease (Figure 3). Overall mean percentage of glomeruli with crescents in rats with EAV was 5.2±1.6% (Figure 4). Consistent with the histological findings, no immune deposits were evident on samples analysed by electron microscopy (data not shown). All EAV rats, but no control rats, developed haematuria
and 72% had albuminuria 6 weeks post immunisation (>1mg/24hr, mean 11.9 ± 4.4mg/24hr, Figure 4).

**Induction of ANCA in WKY rats augments leukocyte adhesion and transmigration induced by CXCL1**

Having demonstrated the presence of ANCA in our rat model of EAV, their effects on leukocyte-endothelial interaction *in vivo* were investigated by intravital microscopy using the mesenteric preparation. For this purpose, leukocyte responses within rat mesenteric venules were observed and quantified in EAV rats in the absence of an inflammatory stimulus and after topical application of the chemokine CXCL1. CXCL1 is a rat homologue of IL-8, a chemokine which is likely to be of pathophysiological relevance in ANCA-associated vasculitis. Intravital microscopy was performed on rats 6-7 weeks after immunisation with hMPO or HSA. Of note, there was no significant difference between the two groups of rats with respect to multiple systemic or microhaemodynamic parameters (Table 1). Furthermore, no significant difference in basal leukocyte rolling flux or leukocyte transmigration was noted between the two groups of rats. Baseline firm adhesion was variable and slightly higher in the HSA-immunised group as compared to the hMPO-immunised group. While the reason for this small difference is unclear, overall the findings do not indicate the occurrence of increased leukocyte-vessel wall interactions in the EAV rats under basal conditions.

In contrast, after topical CXCL1 application, a significant increase in both leukocyte firm adhesion and transmigration was observed in hMPO-immunised compared to HSA-
immunised rats (Figure 5). Sixty minutes after application of the chemokine, adhesion was increased by 32% in the hMPO-immunised group (p<0.01), a response that was accompanied by a 65% increase in transmigration at 90 minutes (p<0.001). There was no correlation between the severity of glomerular injury and the intravital microscopy findings. Thus, in an acute inflammatory milieu, as induced in the present study by local application of the chemokine CXCL1, hMPO-immunised rats, with high titres of circulating ANCA, exhibited enhanced microvascular inflammatory responses of leukocyte adhesion and transmigration. These findings provide in vivo evidence of a potential pathophysiological effect of ANCA in EAV, a model with pathology that very closely mimics human ANCA-associated SVV.

Passive transfer of ANCA-rich Ig confers enhanced microvascular responses to CXCL1 in naive recipient rats

To confirm that the enhanced microvascular responses observed in rats with EAV were due to circulating ANCA, Ig prepared from sera from hMPO- or HSA-immunised rats was transferred to naïve WKY rats prior to quantification of leukocyte responses by intravital microscopy. The protocol used was similar to that described above, with the addition of a 30 minute quantification period following Ig injection prior to mesenteric superfusion with CXCL1 or Tyrode’s solution.

Injection of either EAV or control Ig (20mg/kg) had no effect on baseline systemic or microhaemodynamic variables (Table 1). Before Ig infusion, adhesion and transmigration levels were not significantly different between the two groups. However,
following i.v. injection of EAV Ig, but not control Ig, leukocyte firm adhesion was significantly enhanced in rats treated with topical Tyrode’s solution (mean adhesion at 120 minutes after EAV and control Ig were 6.6 ± 2.1 and 0.6 ± 0.2 leukocytes/100µm segment, respectively; n=3-4, p<0.001; Figure 6A). Interestingly, this increased adhesion response was not accompanied by an increase in leukocyte transmigration (Figure 6B). These results demonstrate that using the present experimental protocol, ANCA-rich Ig on its own can induce leukocyte firm adhesion to venular walls, though this enhanced leukocyte/endothelial cell interaction does not lead to leukocyte transmigration. Hence, in the light of our findings in the active immunisation model, we next investigated the effect of i.v. ANCA-rich Ig on leukocyte responses induced by topical CXCL1. In agreement with our previous results, topical administration of this chemokine elicited time-dependent increases in both leukocyte firm adhesion and transmigration in rats injected with control Ig, as compared to baseline values (Figure 6C and 6D). Both of these responses were enhanced in rats receiving ANCA-rich Ig, with the transmigration response at 90 minutes post application of CXCL1 being 79% greater in rats injected with EAV Ig, as compared to rats injected with control Ig.

Taken together, these findings demonstrate that the MPO-ANCA induced in the EAV rat model of SVV have a biological effect on leukocyte adhesion and transmigration in vivo.

To address the association of ANCA with vascular injury, the experimental models detailed above were extended to enable direct investigation of microvascular injury resulting in haemorrhage.
Transfer of ANCA can induce microvascular haemorrhage

Patients with SVV develop microvascular occlusion and destruction with consequent haemorrhage into surrounding tissue. This is manifested by purpura, lung haemorrhage and bleeding into other organs. Hence, the effect of ANCA on the haemorrhagic response to an inflammatory stimulus was of particular interest. To quantify this variable by intravital microscopy, two methods were employed, governed by the extent of the response. In actively immunised rats, where the haemorrhagic response was relatively extensive and focal sites of haemorrhage could be seen by naked eye (Figure 7A), or by histological analysis (Figure 7B), the response was quantified as a fraction of responding venular segments. Using this approach, a trend towards an increase in haemorrhagic response following superfusion with CXCL1 was observed in hMPO-immunised rats (17.1%, interquartile range 0-37 in EAV v 0%, interquartile range 0-15 in control rats, p=0.09, Figure 7C). For quantification of minor degrees of haemorrhage, as observed in the passive transfer experiments, a global visual-analogue score (0-5) was used to assess the severity of haemorrhage in the mesentery as a whole. Using this method, recipients of ANCA-rich Ig showed a significant increase in microvascular haemorrhagic response following topical application of CXCL1 (Figure 7D), with a trend towards spontaneous haemorrhage following superfusion with Tyrode’s solution alone. Real time development of ANCA-induced microvascular haemorrhage is depicted in Supplemental Video 1.
Discussion

The presence of ANCA is implicated in the pathogenesis of SVV and has been shown in murine models to be sufficient to cause pauci-immune glomerulonephritis. However, to date, there has been no in vivo evidence directly associating these antibodies with leukocyte-mediated vascular damage. Using a novel rat model of ANCA-driven EAV, which has many similarities to human ANCA-associated vasculitis, our findings provide the first direct evidence for the ability of ANCA to enhance leukocyte-vessel wall interactions in vivo. Specifically, rats with EAV exhibited an exaggerated adhesion and transmigration response to a pathophysiologically relevant inflammatory stimulus, CXCL1, as observed by intravital microscopy. This effect was transferable to naïve rats by transfer of Ig, proving that the effect was due to circulating ANCA. Indeed, ANCA on its own, without any additional topical stimulus, was capable of inducing firm adhesion of leukocytes to the vascular endothelium. Furthermore, our study provides evidence to support the concept that ANCA-induced exaggerated leukocyte responses can lead to vascular injury, as manifested by the development of microvascular haemorrhage. It is possible that these are early events in the cascade leading to necrotising vasculitis.

WKY rats immunised with hMPO develop a polyclonal antibody response to this antigen that is accompanied by the development of pauci-immune focal necrotising crescentic glomerulonephritis and pulmonary haemorrhage. This animal model of autoimmune SVV is similar in many respects to the analogous human disease and provides a unique opportunity to study the effects of ANCA in vivo. Specifically, the glomerular lesion seen, though relatively mild, is pauci-immune in nature, as demonstrated by
immunofluorescence and electron microscopy, i.e. there are only scant immune deposits present. The EAV model, like human AASV, is primarily a haematuric condition. Although albuminuria is present in the majority of rats with EAV, it is mild (around 3-10mg/24hr) compared to other rat models of glomerulonephritis. This model has some similarities with previous work performed in the early 1990’s. Brouwer et al used heterologous MPO (at a low dose: 10µg / rat) to induce anti-MPO antibodies in Brown Norway rats. These animals developed granulomatous crescentic nephritis in kidneys perfused with lysosomal extract and hydrogen peroxide. While immune deposits were initially felt to be transient in this model, Yang et al found deposits up to day 10. While many researchers now feel that this is a model of immune-complex deposition disease, rather than pauci-immune vasculitis, it provided an important foundation for future work.

Although the protein sequences of rat (accession gi27674721) and human (accession gi4557759) MPO share 85.7% homology, previous studies employing an ELISA-based approach have found that monoclonal anti-hMPO antibodies raised in mice, and sera from patients with AASV, have limited cross reactivity with rat MPO. Because of these reports, and since the binding of rat anti-hMPO antibodies to rat leukocytes was critical to our in vivo functional studies, initial experiments investigated this cross-reactivity. Evidence was provided for the ability of anti-hMPO antibodies raised in rats to bind to rat leukocytes. We believe that the difference between our findings and those in the study by Patry et al relate to two factors. Firstly, in the majority of their experiments, monoclonal antibodies were used, which presumably have activity against a restricted number of epitopes on the MPO molecule. We have induced high titres of polyclonal antibodies,
amongst which there are apparently antibodies that cross-react with epitopes on the rat MPO molecule. Secondly, in the previous study, rat MPO was immobilised on plastic, which may alter the conformational epitopes compared to those found in the cellular systems that we used.

Leukocyte-vessel wall interactions in EAV rats, as compared to HSA-immunised control rats, were investigated in mesenteric venules as observed by intravital microscopy. Using this model, topical application of the chemokine CXCL1 induced a significant increase in leukocyte firm adhesion and transmigration in control rats, compared to rats treated with topical Tyrode’s solution. These responses were significantly enhanced in rats immunised with hMPO, resulting in a 65% increase in leukocyte transmigration in EAV rats 90 minutes post superfusion of the mesenteric tissue with CXCL1. Of note, CXCL1 is a rat homologue of the human chemokine IL-8 (CXCL8), an inflammatory mediator implicated in the pathogenesis of ANCA-associated vasculitis\textsuperscript{18}. The need for a priming factor to observe potentially pathogenic effects of ANCA on neutrophils is consistent with numerous previous \textit{in vitro} studies. Priming with cytokines such as TNF\textgreek{a} has been shown to be necessary to facilitate the stimulation by ANCA of enhanced neutrophil degranulation and neutrophil-dependent endothelial cytotoxicity\textsuperscript{5,24}. In addition, while the microvessels of the mesentery are not identical to those of the glomerular tuft, there is evidence to suggest that endothelial dysfunction seen in AASV is global \textsuperscript{25}. Thus, our findings demonstrate that the presence of ANCA have the capacity to enhance leukocyte-vessel wall interactions as induced by a pathologically relevant inflammatory mediator.
To assess directly the role of circulating ANCA in these observations, ANCA-rich Ig were prepared from the serum of EAV rats and administered to naïve animals. Of note, it is not possible to compare directly the responses observed in these passive transfer experiments to those in the actively immunised animals, as the use of Freund's adjuvant in the latter was associated with enhancement of all leukocyte-endothelial responses. Naïve rats injected intravenously with intravenous ANCA-rich Ig exhibited a rapid and significantly enhanced leukocyte firm adhesion response that was not observed in rats injected with control Ig. Interestingly, this increase in leukocyte firm adhesion was not associated with enhanced transmigration, suggesting that perhaps a second inflammatory stimulus was required to induce the migration of adherent leukocytes through venular walls. Indeed, topical application of CXCL1 to mesenteric tissue of rats previously injected with intravenous ANCA-rich Ig led to a significantly enhanced transmigration response, as compared to responses detected in naïve rats injected with Ig from HSA-immunised rats. Taken together, these findings provide the first direct evidence for the ability of ANCA to enhance leukocyte firm adhesion, a response that under conditions of a local inflammatory milieu (as induced in our model by topical application of CXCL1) can be translated into leukocyte transmigration.

Whilst the precise mechanism by which this occurs in vivo is at present unclear, in vitro studies have provided a number of potential mechanistic indications. For example, the binding of ANCA to leukocytes has been shown to stimulate activation and/or upregulation of β integrins26,27. Indeed, as illustrated by Radford et al, under both static and flow conditions, ANCA can markedly increase neutrophil adhesion to untreated and
stimulated endothelium\textsuperscript{11,28,29} in a $\beta_2$ integrin dependent manner. Hence, we can speculate that, within the \textit{in vivo} scenario of our studies, binding of ANCA to neutrophils renders them primed for an enhanced adhesion response. In the presence of an inflammatory stimulus, such as CXCL1, these effects are then translated into greater levels of firm adhesion and subsequent transmigration. Based on the cited \textit{in vitro} observations, many of these effects may be $\beta_2$ integrin mediated, although the precise adhesive pathways involved \textit{in vivo} are currently unknown and are under investigation in our laboratory.

As exaggerated leukocyte-vessel wall interactions can lead to leukocyte-mediated vascular damage, a characteristic feature of the pathology of SVV, we investigated the effect of ANCA on vascular injury as manifested by local microvascular haemorrhage. For this purpose, to investigate ANCA-induced haemorrhage using intravital microscopy, two quantification procedures were established as governed by the extent of the responses observed. In both models, evidence was obtained for ANCA-induced microvascular haemorrhage, a response that was largely associated with ANCA-induced leukocyte transmigration. These findings are consistent with \textit{in vitro} observations indicating that ANCA can induce degranulation of primed neutrophils, with consequent release of reactive oxygen species and proteolytic enzymes, responses that under conditions of neutrophil adhesion to endothelium could cause endothelial injury\textsuperscript{30}. Hence our findings support the general hypothesis that ANCA-induced exaggerated leukocyte-vessel wall interactions can lead to vascular injury. This is in line with the recent description by Xiao et al\textsuperscript{13} of the induction of SVV in mice by transfer of anti-MPO rich antibodies.
Collectively, using a novel rat model of EAV, the present results provide the first direct evidence for the ability of ANCA to induce leukocyte-vessel wall interactions and leukocyte-mediated vascular damage \textit{in vivo}. We and others have recently defined the role of various treatment modalities for ANCA-associated vasculitis in the clinical arena, such as the duration of cyclophosphamide treatment\textsuperscript{31}, potential roles for anti-TNF biologic therapy\textsuperscript{32} and the indications for antibody removal by plasma exchange\textsuperscript{33}. We now have the opportunity, using animal models, to design and test therapies targeting the specific pathways activated by ANCA. This should lead to improved treatment for patients with SVV.

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Table 1. Mean arterial pressure, leukocyte count, blood flow and vessel diameter in test rats

WKY rats were immunised (“Active immunisation”) with hMPO (EAV, n=7-20 per group) or HSA (Control, n=8-18 per group) in CFA, or received Ig (“Passive transfer”; ANCA+, n=9, ANCA-, n=9). No significant differences between the groups within each experiment were observed. Values are mean ± SEM.

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<td>(x10^6/mL)</td>
<td>mm/sec</td>
<td>µm</td>
</tr>
<tr>
<td><strong>Base</strong></td>
<td><strong>90 min</strong></td>
<td><strong>PMN</strong></td>
<td><strong>PBMC</strong></td>
<td><strong>Base</strong></td>
</tr>
<tr>
<td><strong>Active immunisation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EAV</strong></td>
<td>105.6±3.9</td>
<td>109.8±5.8</td>
<td>1.5 ± 0.1</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>103.4±2.6</td>
<td>106.9±3.0</td>
<td>1.2 ± 0.2</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td><strong>Passive transfer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANCA+</strong></td>
<td>105.4±3.0</td>
<td>121.4±3.7</td>
<td>1.0 ± 0.1</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td><strong>ANCA-</strong></td>
<td>106.0±3.8</td>
<td>118.3±2.7</td>
<td>1.3 ± 0.3</td>
<td>4.0 ± 0.4</td>
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Figure legends

Panel 1

Panel 3

3a: Rat PMN
mean RFI=2.0

3b: Human PMN
mean RFI=6.9

Panel 2

Panel 4

hMPO
WKY
lysate

Human
lysate

EAV Ig
Ctrl Ig
EAV Ig

97
66
MW

α-hMPO antibody status
+  -  -  +  -
Human PMN
+  +  +  +
WKY PMN
+  +  +
WKY sera
Human sera

EAV Ig
Ctrl Ig
EAV Ig

97
66
MW
Figure 1 *Sera from hMPO and HSA-immunised WKY rats bind to WKY and human neutrophils.* Panel 1. Human and WKY rat PMN were indirectly stained with sera (1:20) from hMPO (EAV) or HSA-immunised (control) rats, and sera from patients with AASV and observed by confocal microscopy. Representative of 5 experiments involving 5-13 separate sera. Panel 2. Cells were stained as in panel 1 and fluorescence intensity was quantified using image-Pro plus software. Statistically significant differences are shown by asterisks, **p<0.01 and ***p<0.001. AFU=Arbitrary fluorescence units. Panel 3. Rat leukocytes were incubated with Ig prepared from EAV rats (open curves) or control rats (filled curves). Both surface and intracellular antigens were stained sequentially and rat PMNs were identified based on side scatter profile and binding of anti-HIS48 mAb. Panels 2a and 2b represent cells stained with control Ig (closed curves) or EAV Ig (open curves) on rat and human PMNs respectively. Results are representative of 4 separate experiments. Panel 4. Lysates of WKY rat and human leukocytes were prepared and analysed by Western blotting. Lane 1: Purified hMPO incubated with pooled Ig from rats with EAV. Lane 2: WKY rat leukocyte lysate incubated with pooled Ig from rats with EAV. Lane 3: WKY rat leukocyte lysate incubated with pooled Ig from control rats. Lane 4: Human PMN lysate incubated with pooled Ig from rats with EAV. Separate blots are indicated by vertical lines.
Figure 2. **WKY rats immunised with hMPO develop focal necrotising crescentic glomerulonephritis and pulmonary haemorrhage.** A. No histological abnormalities in a rat immunised with HSA (Periodic acid Schiff); B. Severe crescentic glomerulonephritis (arrows) in a rat immunised with hMPO (Periodic acid Schiff); C. Tubulo-interstitial nephritis with red cell cast (arrow) in a rat immunised with hMPO (H&E); D. Focal glomerular necrosis, with an adjacent glomerulus showing normal architecture in a rat immunised with hMPO (H&E); E. Focal necrotising lesion with early crescent formation (arrow) in a rat immunised with hMPO (H&E); F. Circumferential glomerular crescent (arrow) in a rat immunised with hMPO (H&E); G. Normal lung architecture in a rat immunised with HSA (H&E). H. Pulmonary vasculitis (arrow) in a rat immunised with hMPO (H&E); I. Pulmonary haemorrhage in a rat immunised with hMPO, with extensive blood in the alveolar spaces (H&E).
Figure 3. **Glomerulonephritis in EAV is pauci-immune.** Glomerular Ig deposition was assessed using direct immunofluorescence on 3µm frozen sections. A. Linear staining for IgG in a rat with experimental autoimmune glomerulonephritis, a model of anti-glomerular basement membrane disease. This serves as a positive control; B. Scanty Ig deposits in a rat with EAV; C. Negative staining in a rat immunised with CFA alone. Images were captured using a confocal microscope and bars represent 100µm.
Figure 4. **EAV is characterised by haematuria, low-grade albuminuria and focal crescent formation.** Six weeks after immunisation, haematuria (A) was assessed by dipstick and albuminuria (B) by ELISA. C. The percentage of glomeruli with crescents was quantified on Periodic acid Schiff sections. ***p<0.0001 comparing proteinuria between EAV and control rats. No statistical comparison is possible in panels A and C as all values in the control group are zero.
Figure 5. Adhesion and transmigration profiles in WKY rats actively immunised with hMPO or HSA in response to topical CXCL1. Intravital microscopy was performed on rats 6-7 weeks post immunisation with hMPO (EAV rats) or HSA (control rats). After recording baseline responses of adhesion (A) and transmigration (B), topical CXCL1 (3 x 10^{-9}M) or Tyrode’s balanced salt solution was superfused over the mesentery and responses quantified for a further 90 minutes, during which time superfusion of the topical agent was maintained. The data represent mean ± SEM, n=11-13 separate rats per group for CXCL1 and n=6-7 per group for Tyrode’s. Statistically significant differences between different groups of rats are shown by asterisks, *p<0.05 and ***p<0.001.
Figure 6. Passive transfer of ANCA-rich immunoglobulin enhances microvascular responses in naïve WKY rats. Intravital microscopy was performed on naive WKY rats following i.v. administration of 20mg/kg of pooled anti-hMPO positive Ig (squares) or control Ig (triangles) and leukocyte responses of adhesion (A, C) and transmigration (B, D) were measured for up to 2 hours. In some rats, after 30 minutes, the Tyrode’s mesenteric superfusion was changed to superfusion with 3 x 10^{-9}M CXCL1 (C, D), which was maintained for a further 90 minutes. The data represent the mean ± SEM, n=5 separate rats in CXCL1 groups and n=3-4 in Tyrode’s groups. Statistically significant differences between groups of rats are shown by asterisks, *p<0.05 **p<0.01 and ***p<0.001.
Figure 7. **ANCA induce post-capillary venular haemorrhage.** Intravital microscopy was performed on WKY rats 6-7 weeks after immunisation with hMPO (EAV rats) or HSA (control rats) (C) or on naïve WKY rats with infusion of ANCA-rich or ANCA-negative Ig (D). A representative macroscopic image of petechiae around a mesenteric arcade is shown in panel A. The microscopic appearance of this is shown in panel B (H&E stain, x20). In the active immunisation model (C), superfusion with $3 \times 10^{-9}$M CXCL1 was maintained for 90 minutes and haemorrhage was quantified by expressing the number of haemorrhagic venular segments as % of total segments studied at each time point. The data represent the median, interquartile range (box) and range (error bars, n=11 in control group and 13 in EAV group). Of note, although minor degrees of haemorrhage were seen in the control group, the median remained at zero throughout the
experiment. In the passive transfer model (D) superfusion fluid was changed in some experiments from Tyrode’s solution to CXCL1 30 minutes after Ig infusion and haemorrhage was quantified after a further 90 minutes using a global mesenteric visual / analogue score (n=4-10 separate rats in each group). Statistically significant differences between groups of rats are shown by asterisks, *p<0.05.
Anti-neutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions in vivo

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