Extended report

Effects of p38 mitogen-activated protein kinase inhibition on anti-neutrophil cytoplasmic autoantibody pathogenicity in vitro and in vivo

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ABSTRACT

Objective To determine whether inhibition of p38 mitogen-activated protein kinase (p38MAPK) reduces the pathogenicity of anti-neutrophil cytoplasmic autoantibodies (ANCAs) in vitro and in vivo.

Methods The effects of the p38MAPK-specific inhibitor AR-447 were studied in vitro using neutrophil respiratory burst and degranulation assays, and in lipopolysaccharide (LPS)-stimulated human glomerular endothelial cells. In vivo, p38MAPK inhibition was investigated in a mouse anti-myeloperoxidase (MPO) IgG/LPS glomerulonephritis model. Mice were treated orally with AR-447 daily, starting before (pretreatment group) or 24 h after disease onset (treatment group), and killed after 1 or 7 day(s).

Results In vitro, AR-447 diminished neutrophil respiratory burst and degranulation induced by patient-derived MPO-ANCA and proteinase 3 (Pr3)-ANCA. In glomerular endothelial cells, AR-447 reduced LPS-induced secretion of IL-6 and IL-8, but not of MCP-1. In mice, pretreatment with AR-447 reduced albuminuria 1 day after induction of glomerulonephritis. After 7 days, no effects on urinary abnormalities were observed upon AR-447 pretreatment or treatment. Also, glomerular neutrophil accumulation was not diminished. In contrast, glomerular macrophage accumulation and the formation of glomerular crescents was significantly reduced by AR-447 pretreatment (vehicle: $12.5 \pm 5.6\%$ crescentic glomeruli; AR-447: $7.7 \pm 2.7\%$) and treatment (vehicle $14.6 \pm 1.8\%$; AR-447 $6.0 \pm 3.4\%$) at 7 days.

Conclusion This study shows that p38MAPK inhibition markedly reduces ANCA-induced neutrophil activation in vitro. In vivo, p38MAPK inhibition partly reduced crescent formation when the drug was administered prior to disease induction and after disease onset, suggesting that besides p38MAPK activity other signalling pathways contribute to the disease activity.

INTRODUCTION

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated small vessel vasculitides are systemic inflammatory diseases affecting small-sized to medium-sized blood vessels.[1] Patients with ANCA-associated small vessel vasculitis present with circulating autoantibodies directed against the neutrophilic enzymes myeloperoxidase (MPO) or proteinase 3 (Pr3). Involvement of the kidney is common in patients with ANCA-associated small vessel vasculitis, resulting in focal segmental crescentic glomerulonephritis. In mice, administration of anti-MPO antibodies causes crescentic

glomerulonephritis and vasculitis, demonstrating the pathogenic potential of ANCAs.[2]

Evidence derived from clinical, in vitro and animal model studies support the concept that in ANCA-associated glomerulonephritis, ANCAs synergise with infection-derived proinflammatory stimuli in causing full blown disease.[1] Proinflammatory stimuli, such as tumour necrosis factor α (TNFα), cause upregulation of adhesion molecules on endothelial cells and neutrophils, promoting leucocyte–endothelial cell interactions. At the same time, proinflammatory cytokines prime neutrophils to express the ANCA target antigens, MPO and Pr3, on the cell surface, making these accessible for interaction with ANCAs.[3] Binding of ANCAs to MPO or Pr3 activates the neutrophil, via Fcγ receptor ligation, to degranulate and to undergo respiratory burst, causing endothelial cell damage. Thus, disease induction in ANCA-associated glomerulonephritis most likely involves inflammatory cells, in particular neutrophils, and intrinsic renal cells, such as glomerular endothelial cells.

One of the inflammatory signalling pathways that is suggested to be involved in ANCA-mediated neutrophil activation is the p38 mitogen-activated protein kinase (p38MAPK) pathway.[4] p38MAPK becomes activated by stress-related signals, such as proinflammatory cytokines, through phosphorylation mediated by upstream kinases. Activated p38MAPK activates transcription factors that regulate transcription of inflammation-related genes either directly by phosphorylating transcription factors or indirectly by phosphorylating downstream kinases, for example, MAPK-activated protein kinase 2 (MAPKAPK-2). p38MAPK is activated in several inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel disease.[5] [6] It has been shown that inhibition of p38MAPK activation markedly reduces ANCA-mediated neutrophil activation in vitro.[4] Furthermore, recent studies have demonstrated that p38MAPK is activated in glomerular lesions of patients with ANCA-associated glomerulonephritis.[7] In these lesions, active p38MAPK was predominantly observed in inflammatory cells and in podocytes. Taken together, these observations suggest that inhibition of p38MAPK may be of benefit for the treatment of ANCA-associated vasculitis.

The aim of this study was to determine whether inhibition of p38MAPK reduces the pathogenicity of ANCAs in vitro and in vivo. To this end,

we analysed whether the p38MAPK-specific inhibitor AR-447 reduced ANCA IgG-induced neutrophil activation and lipopolysaccharide (LPS)-induced production of cytokines by glomerular endothelial cells and podocytes in vitro. In addition, we analysed the effects of AR-447-mediated p38MAPK inhibition on development of renal injury in the mouse model of anti-MPO IgG/LPS-induced glomerulonephritis.

MATERIALS AND METHODS

Drugs

p38MAPK inhibitors AR-447 and RWJ-67657 (4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyn-1-ol) were provided by Array BioPharma (Boulder, Colorado, USA) and Johnson & Johnson Pharmaceutical R&D (Raritan, New Jersey, USA), respectively. AR-447 is a selective p38MAPK inhibitor with low nanomolar potency against the α (IC_{50}=21 nM) and β (IC_{50}=84 nM) isoforms of the enzyme. AR-447 exerts its inhibiting effect on p38MAPK by binding to the ATP-binding pocket of p38MAPK, thereby inhibiting its ability to phosphorylate downstream targets (such as MAPKAPK-2). In human whole blood assays and murine macrophages, AR-447 inhibits LPS-induced TNFα production with IC_{50}s in the 70–100 nM range ex vivo.[8] AR-447 is also well tolerated for multiple days of dosing in rodents and possesses a good pharmacokinetic profile (data not shown). RWJ-67657 also inhibits the enzymatic activity of p38MAPK α and β isoforms and has no significant activity against a variety of other enzymes.[9][10] For in vitro experiments, AR-447 and RWJ-67657 stock solutions (10 mM) were prepared in dimethylsulfoxide (DMSO). For in vivo experiments, AR-447 was suspended in 1% carboxymethylcellulose/0.5% Tween-80 (CMC/Tween).

Neutrophil activation assays

Neutrophils were isolated from healthy donors and analysed for ANCA IgG-induced respiratory burst and degranulation as described previously.[11] In short, before priming of the neutrophils with 2 ng/ml recombinant human TNFα (Boehringer, Ingelheim, Germany) for 15 min at 37°C, cells were pretreated with p38MAPK inhibitor or vehicle (DMSO) for 30 min at 37°C at the indicated concentrations. Next, the neutrophils were incubated with monoclonal antibodies directed against MPO (10 µg/ml; 266.6K1; IQ Products, Groningen, The Netherlands) or Pr3 (5 µg/ml, Pr3G-2; Hycult Biotechnology, Uden, The Netherlands) or with patient-derived ANCA IgG (200 µg/ml) for 1 h at 37°C. Respiratory burst was determined in dihydrorhodamine-123 (DHR123)-loaded cells by oxidation of the non-fluorescent DHR123 to the fluorescent rhodamine-123. Mean fluorescence intensity (MFI) of rhodamine-123 was measured by flow cytometry and data are expressed as % activation=($MFI_{inhibitor}$/MFI_{DMSO})×100. The lactoferrin content in the supernatant was measured by ELISA, as described previously.[12]

Culture and stimulation of glomerular cells

Human conditionally immortalised glomerular endothelial cells[13] were cultured as described previously.[14] Human conditionally immortalised podocytes[15] were cultured in RPMI (Cambrex-Lonza, Breda, The Netherlands) supplemented with 10% fetal calf serum (HyClone, Logan, Utah, USA) and insulin-transferrin-selenium (Gibco, Invitrogen, Breda, The Netherlands). Both cell lines were propagated at 33°C (when cells have an immortalised phenotype), whereas experiments were carried out after 5–8 days of incubation at 37°C (non-proliferative phenotype). Cells were pretreated with 10 µM AR-447 (or DMSO 0.1% as vehicle control) for 30 min and stimulated with 1 µg/ml LPS (*Escherichia coli*, serotype O26:B6; Sigma, St Louis, Missouri, USA) for the indicated time periods.

Western blotting

To analyse p38MAPK and MAPKAPK-2 phosphorylation upon stimulation, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 0.01% bromophenol blue) and lysates were used for western blotting. Blots were incubated with phosphospecific antibodies to p38MAPK (1:400; Cell Signalling, Beverly, Massachusetts, USA) and MAPKAPK-2 (1:400; Cell Signalling). Detection was performed with horseradish peroxidase-conjugated secondary antibody (1:1000; DakoCytomation, Carpinteria, California, USA) and chemoluminescence (Pierce, Rockford, Illinois, USA). Total p38MAPK and MAPKAPK-2 protein expression levels were analysed to confirm equal loading.

Cytokine measurements in glomerular cells

RNA was isolated using RNeasy Plus Mini kit (Qiagen, Benelux, Venlo, The Netherlands). Reverse transcription was carried out using Superscript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Gene expression was measured using primer–probe sets specific for human interleukin (IL)-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), monocyte chemoattractant protein (MCP)-1 (Hs00234140_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) on an ABI Prism 7900HT Sequence Detection System (all from Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Relative mRNA levels were calculated as $2^{-\Delta CT}$, in which ΔCT is $CT_{gene\ of\ interest}-CT_{GAPDH}$. Protein levels of IL-6, IL-8 and MCP-1 were determined using in-house-developed sandwich ELISAs based on coating with monoclonal antibodies and detection with biotinylated polyclonal antibodies against human IL-6, IL-8 and MCP-1 (all from R&D Systems Europe, Abingdon, UK).

Animals

$Mpo^{-/-}$ mice were backcrossed to a C57BL/6 background for seven generations.[16] Female C57BL/6 wild-type mice were purchased from Harlan (Horst, The Netherlands). All animal experiments were performed according to national guidelines and upon approval of the Institutional Animal Care and Use Committee of the University of Groningen.

Induction and evaluation of anti-MPO IgG/LPS-induced glomerulonephritis

Polyclonal anti-MPO IgG was obtained by isolation of total IgG from pooled sera of murine MPO-immunised $Mpo^{-/-}$ mice as described previously.[17] Crescentic glomerulonephritis was induced in wild-type C57BL/6 mice (aged 8–10 weeks) by intravenous injection of anti-MPO IgG (50 µg/g body weight), followed by intraperitoneal injection of LPS (150 EU/g body weight; *E coli*, serotype O26:B6) 1 h later. Mice (n=5–6 per group) received daily treatment with AR-447 (30 mg/kg) or vehicle (CMC/Tween) via oral gavage, starting either 2 h before (pretreatment) or 24 h after (treatment) anti-MPO IgG administration, and were killed after 1 or 7 day(s). Urine samples were tested for haematuria (0–4+ score) by Combur-Test strips (Roche Diagnostics, Almere, The Netherlands) and albuminuria by ELISA (Bethyl Laboratories, Montgomery, Texas, USA). In kidney sections, the number of glomerular crescents
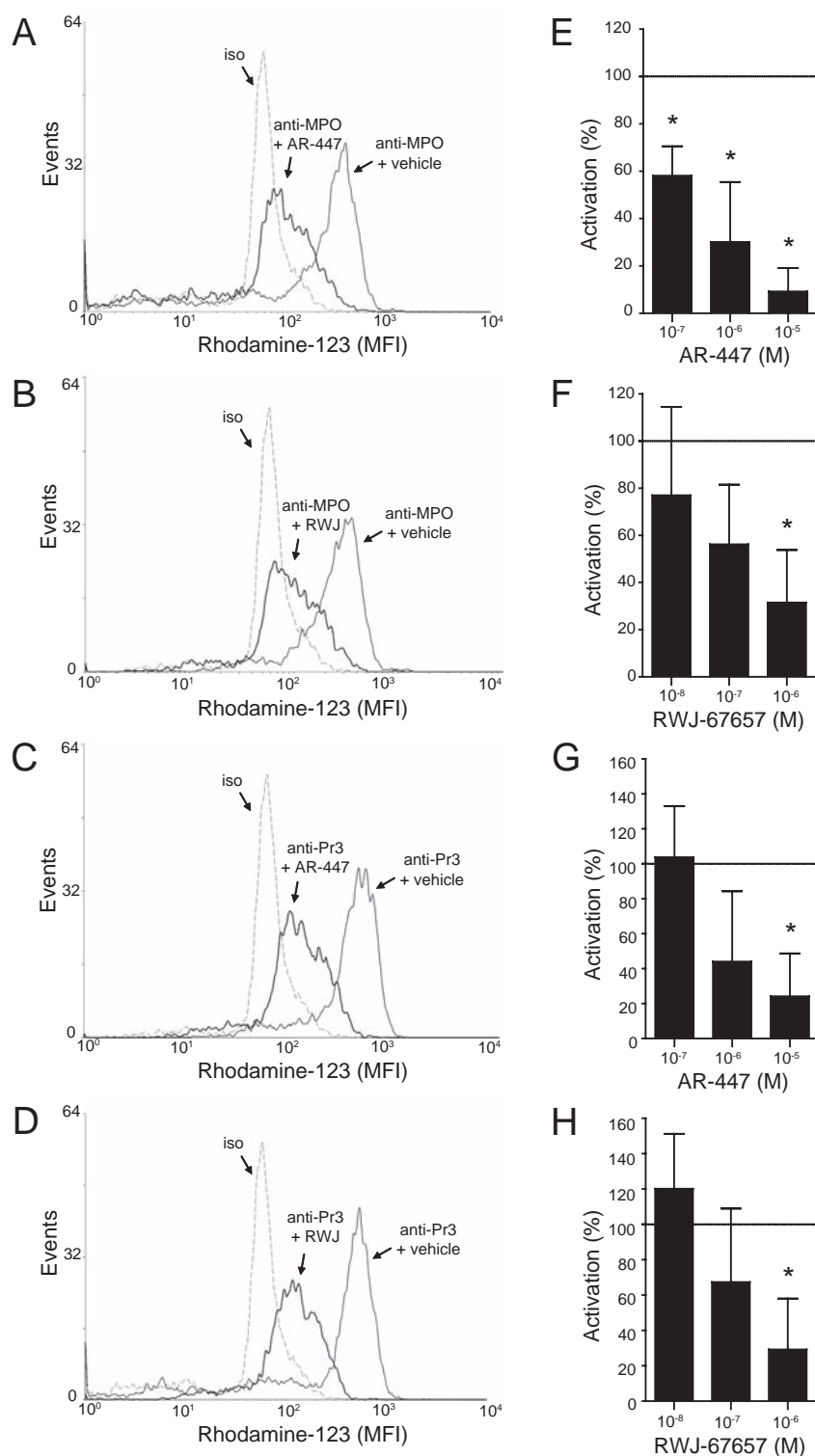
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Figure 1 Inhibition of p38 mitogen-activated protein kinase (p38MAPK) reduces neutrophil respiratory burst induced by anti-myeloperoxidase (anti-MPO) and anti-proteinase 3 (anti-Pr3) monoclonal antibodies (moAb). Respiratory burst was measured in human neutrophils from healthy donors by the conversion of dihydrorhodamine (DHR)-123 into the fluorescent rhodamine-123. Representative histograms of respiratory burst measurements are shown in A–D, demonstrating inhibition of anti-MPO moAb-induced neutrophil respiratory burst pretreatment with p38MAPK inhibitors AR-447 (10 μM, A) and RWJ-67657 (1 μM, B) and inhibition of anti-Pr3 moAb-induced respiratory burst upon pretreatment with AR-447 (10 μM, C) and RWJ-67657 (1 μM, D). Pretreatment with different concentrations of AR-447 (E, G) and RWJ-67657 (F, H) revealed a dose-dependent inhibition of anti-MPO moAb (E, F) and anti-Pr3 moAb (G, H) induced respiratory burst. Bars represent mean ($\pm$SD) percentage of respiratory burst activation of three independent experiments. For every combination of inhibitor concentration and antibody, the appropriate vehicle control was set to 100% activation of respiratory burst. *p < 0.05 compared to vehicle control. Iso, isotype-matched control moAb; MFI, mean fluorescence intensity.
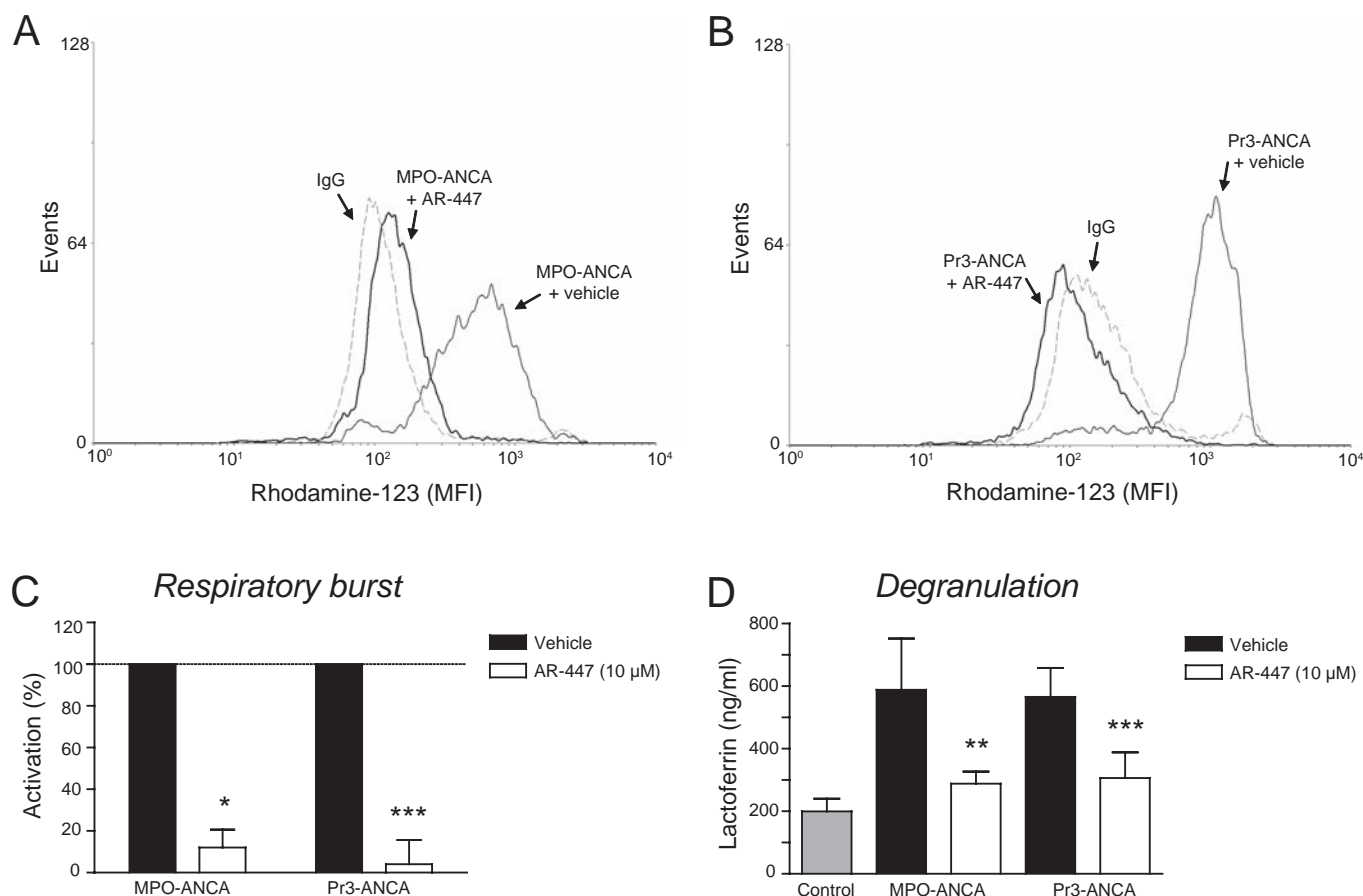
Figure 2 Inhibition of p38 mitogen-activated protein kinase (p38MAPK) reduces anti-neutrophil cytoplasmic autoantibody (ANCA) IgG-induced neutrophil respiratory burst and degranulation. Representative histograms of rhodamine-123 measurements show that inhibition of p38MAPK with 10 μM AR-447 diminished respiratory burst induced upon patient-derived myeloperoxidase (MPO)-ANCA IgG (A) and proteinase 3 (Pr3)-ANCA IgG (B) in neutrophils from healthy donors. C. The mean ($\pm$SD) percentage of respiratory burst activation induced by four MPO-ANCA and four Pr3-ANCA IgG fractions measured in two donors. Respiratory burst induced by ANCA IgG in neutrophils pretreated with vehicle was set to 100%. *p<0.05, ***p<0.001 compared to vehicle control. D. ANCA-induced neutrophil degranulation was determined by measuring the lactoferrin concentration in the supernatant. Pretreatment with AR-447 reduced ANCA-induced lactoferrin release. Bars represent mean $\pm$ SD of four MPO-ANCA and four Pr3-ANCA preparations measured in two donors. **p<0.01, ***p<0.001 compared to vehicle pretreatment.

($\geq$2 cell layers in Bowman's space) was determined by evaluating 100 consecutive glomerular cross-sections in a blinded fashion. Immunohistochemical staining for neutrophils and macrophages was performed using rat anti-mouse Ly6G (clone 1A8; BD Biosciences, Breda, The Netherlands), rat anti-mouse CD68 (clone FA11; AbD Serotec, Oxford, UK) and a peroxidase-based EnVision+ System (DakoCytomation).

Statistical analysis

Statistical significance was determined by paired (neutrophil activation assays) or unpaired one-tailed Student t test using GraphPad Prism V.4.03 (GraphPad Software, San Diego, California, USA).

RESULTS

Inhibition of p38MAPK reduces ANCA IgG-induced neutrophil activation in vitro

To confirm that inhibition of p38MAPK reduces ANCA IgG-induced neutrophil activation in vitro, we employed a p38MAPK-specific inhibitor in the DHR123 oxidation assay and in a degranulation assay (lactoferrin release). Pretreatment with AR-447 decreased neutrophil respiratory burst induced by monoclonal antibodies against MPO and Pr3 in a dose-dependent

manner (figure 1). The extent of inhibition by AR-447 was comparable to that of the p38MAPK inhibitor RWJ-67657. In addition to respiratory burst induced by monoclonal antibodies, pretreatment with 10 µM AR-447 also markedly reduced neutrophil respiratory burst and degranulation induced by ANCA IgG derived from patients with ANCA-associated glomerulonephritis (figure 2). These results confirm that inhibition of p38MAPK reduces ANCA IgG-induced neutrophil activation in vitro.

Inhibition of p38MAPK decreases LPS-induced cytokine production by glomerular endothelial cells in vitro

To analyse whether p38MAPK inhibition affects the LPS-induced production of cytokines by intrinsic glomerular cells, we pretreated human glomerular endothelial cells and podocytes with AR-447 prior to LPS exposure and analysed mRNA levels and protein production of IL-6, IL-8 and MCP-1. Stimulation of glomerular endothelial cells and podocytes with LPS increased phosphorylation of p38MAPK, indicating activation of p38MAPK (figure 3A). In addition, activation of p38MAPK resulted in phosphorylation of its downstream kinase MAPKAPK-2. Phosphorylation of MAPKAPK-2 was fully prevented upon pretreatment with AR-447, demonstrating effective inhibition of p38MAPK activity (figure 3B). In line
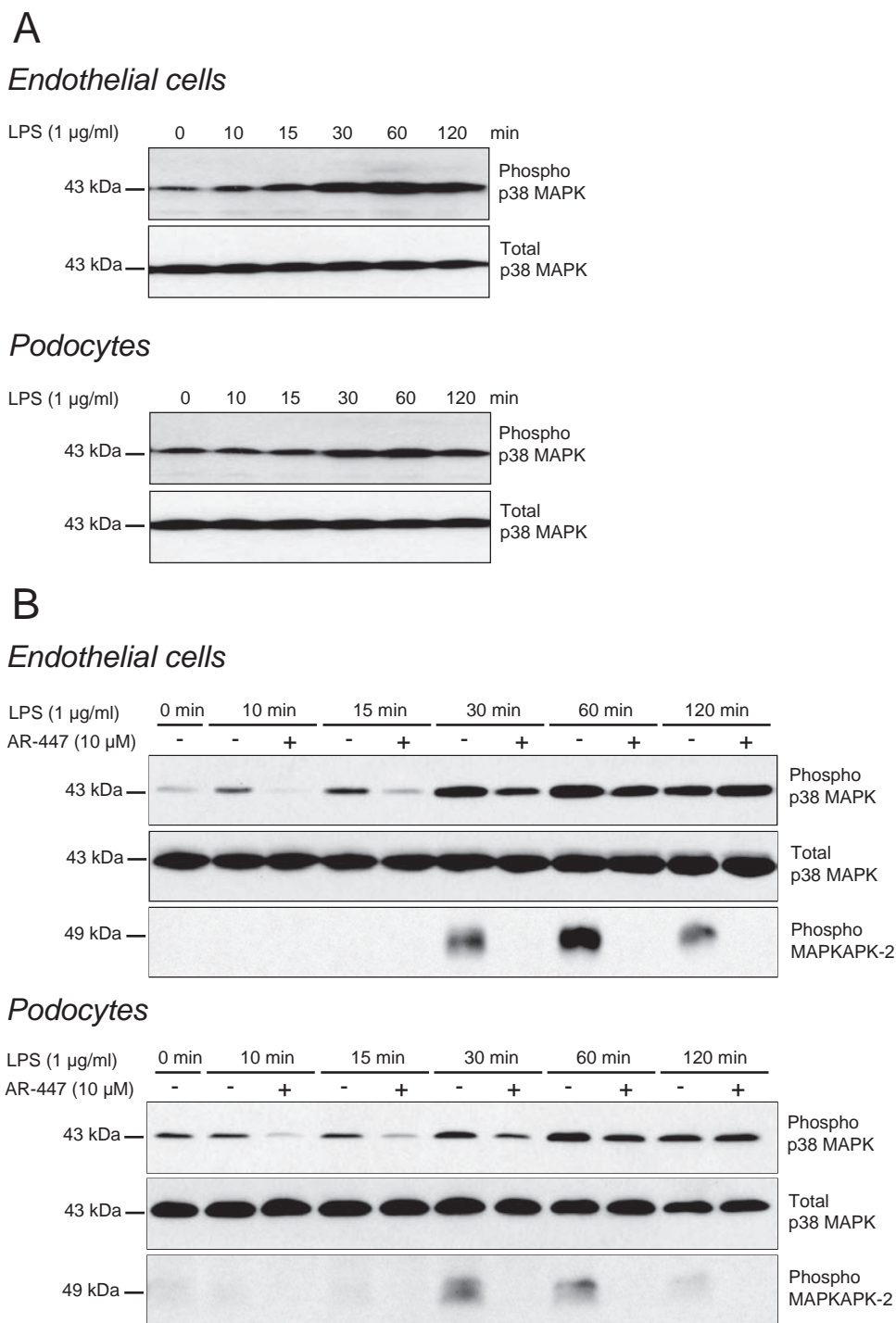
Figure 3 Lipopolysaccharide (LPS)-induced p38 mitogen-activated protein kinase (p38MAPK) activity in glomerular endothelial cells and podocytes is abrogated upon AR-447 treatment. A. Human conditionally immortalised glomerular endothelial cells and podocytes were stimulated with LPS (1 μg/ml) for the indicated times. Cell lysates were analysed for phosphorylated p38MAPK by western blotting. B. Endothelial cells and podocytes were pretreated with either p38MAPK inhibitor AR-447 (10 μM) or with vehicle (dimethylsulfoxide (DMSO)) for 30 min and then stimulated with LPS (1 μg/ml) for the indicated times. Cell lysates were analysed by western blotting for phosphorylation of p38MAPK and phosphorylation of MAPK-activated protein kinase 2 (MAPKAPK-2), a p-p38MAPK downstream target.

with its specificity for p38MAPK, AR-447 did not inhibit phosphorylation of the kinases Akt and extracellular signal-regulated kinase (ERK)1/2 (supplementary figure 1). Next, we analysed whether AR-447 could reduce LPS-induced cytokine production in glomerular endothelial cells and podocytes. In glomerular endothelial cells, AR-447 pretreatment reduced LPS-induced IL-6 and IL-8, but not MCP-1, mRNA expression and protein release (figure 4). In podocytes, production of IL-6 and IL-8 was not increased upon LPS, whereas LPS-induced production of MCP-1 was not inhibited by AR-447 pretreatment (supplementary figure 2). Thus, inhibition of p38MAPK reduces the LPS-induced production of IL-6 and IL-8 by glomerular endothelial cells in vitro, while not affecting MCP-1 expression in either cell type studied.
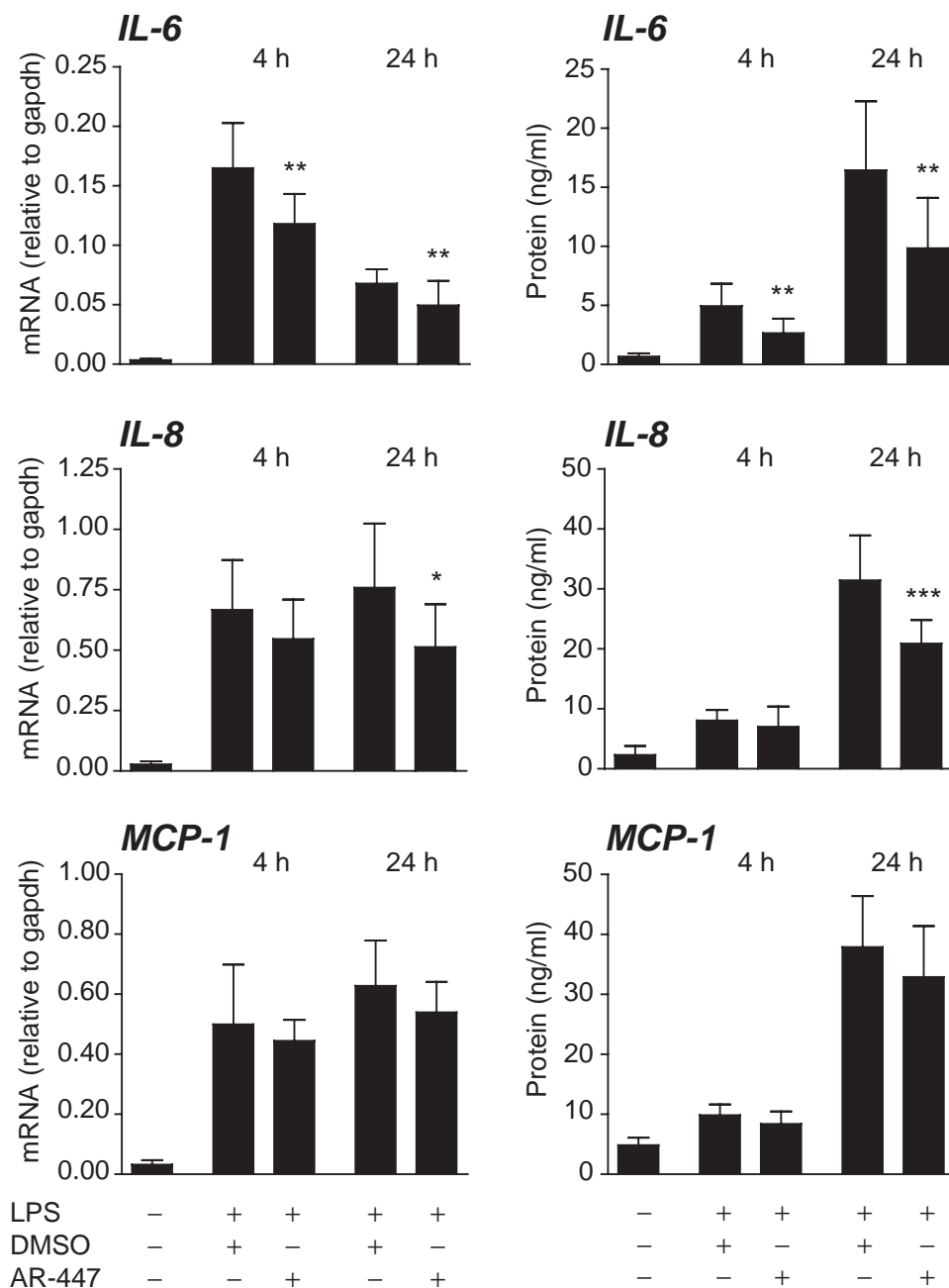
Figure 4 Effect of p38 mitogen-activated protein kinase (p38MAPK) inhibition on lipopolysaccharide (LPS)-induced production of interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in glomerular endothelial cells. IL-6, IL-8 and MCP-1 mRNA levels in the cells and protein levels in the medium upon pretreatment of human glomerular endothelial cells with AR-447 (10 μM) or vehicle (dimethylsulfoxide (DMSO)) for 30 min and stimulation with LPS (1 μg/ml) for 4 or 24 h. Bars represent mean ± SD of 3–4 independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle pretreatment and LPS stimulation at the same timepoint.

Inhibition of p38MAPK reduces glomerular crescent formation in experimental anti-MPO IgG/LPS-mediated glomerulonephritis

Next, we investigated the effect of p38MAPK inhibition on development of renal injury in the mouse model of anti-MPO IgG/LPS-induced glomerulonephritis. Mice receiving anti-MPO IgG and LPS were treated with AR-447 according to either a pretreatment or a treatment protocol, to study the effect of p38MAPK inhibition during the initial glomerular inflammation and the progression to crescentic glomerulonephritis and to evaluate the potential of AR-447 to treat established disease.

In the pretreatment protocol, AR-447 reduced albuminuria 1 day after induction of glomerulonephritis (figure 5A). The

glomerular neutrophil influx however was not altered by AR-447 pretreatment at this time point (figure 6A). After 7 days, albuminuria in mice pretreated with AR-447 did not differ from mice pretreated with vehicle (figure 5). AR-447 also did not reduce haematuria at days 1 and 7. Similarly, in the treatment protocol, AR-447 did not decrease albuminuria and haematuria after 7 days. Renal mRNA levels of the cytokines CXCL1 and CXCL2 (murine homologues of human IL-8), IL-6 and MCP-1 were also not decreased upon AR-447, except for MCP-1 after 7 days in the treatment protocol (supplementary figures 3 and 4). In contrast, pretreatment and treatment with AR-447 reduced the number of glomerular crescents and the number of glomerular macrophages after 7 days (figure 6B,C).
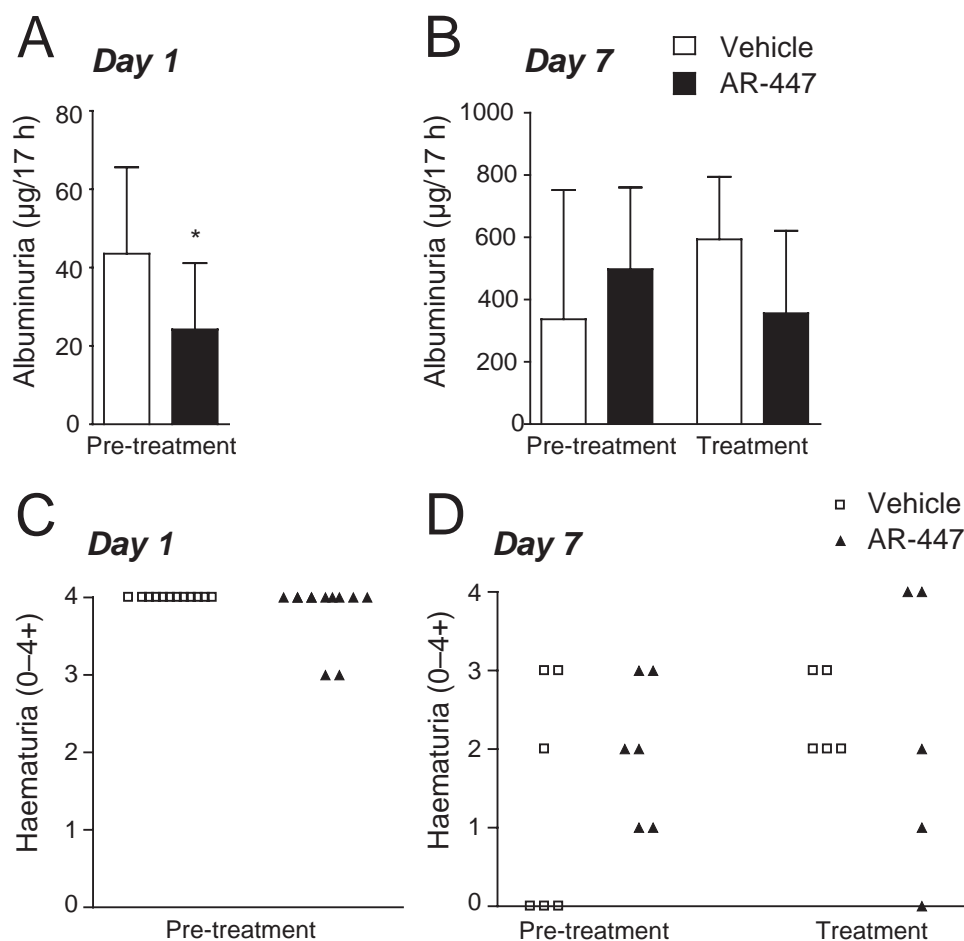
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Figure 5 Effect of AR-447 (pre)treatment on haematuria and albuminuria in mice subjected to anti-myeloperoxidase (MPO) IgG/lipopolysaccharide (LPS)-induced glomerulonephritis. Glomerulonephritis was induced in mice by an intravenous injection of mouse anti-MPO IgG (50 µg/g) followed by an intraperitoneal injection of LPS (150 EU/g). AR-447 (30 mg/kg) or vehicle (1% carboxymethylcellulose/0.5% Tween-80 (CMC/Tween)) was administered daily by oral gavage, starting with the first administration either 2 h before (=pretreatment) or 24 h after (=treatment) anti-MPO IgG administration. Albuminuria (A,B) and haematuria (C,D) were measured at 1 day (A,C) and 7 days (B,D) after induction of glomerulonephritis. Baseline levels of albuminuria (measured 1 week before glomerulonephritis induction) were 19.28 ± 8.80 µg/17 h. Bars represent mean $\pm$ SD of 5–6 mice. *$p < 0.05$ compared to vehicle pretreated mice.

Thus, inhibition of p38MAPK partially reduces development of renal injury in this mouse model of anti-MPO IgG/LPS-induced glomerulonephritis.
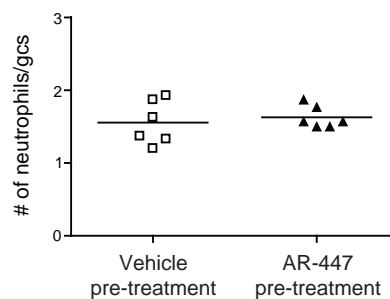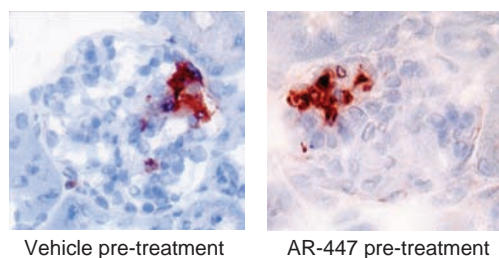
DISCUSSION
In this study, we show that inhibition of p38MAPK reduces pathogenicity of ANCAs in vitro, as shown by a marked attenuation of ANCA IgG-induced respiratory burst and degranulation in human neutrophils. In contrast, inhibition of p38MAPK only partially reduces LPS-induced cytokine production by human glomerular endothelial cells and has a moderate beneficial effect on disease severity in the mouse model of anti-MPO IgG/LPS-induced glomerulonephritis.

Our finding that p38MAPK inhibition abrogated ANCA IgG-induced neutrophil activation confirms and extends previous observations that have demonstrated a role for p38MAPK in this process.[4] In that study, the well described p38MAPK inhibitor SB203580 was shown to reduce ANCA IgG-induced respiratory burst, by preventing translocation of ANCA antigens to the neutrophil membrane. However, concerns have been raised regarding the specificity of SB203580 at the concentration used by the authors (50 µM). At 50 µM, SB203580 can inhibit the activity of other kinases as well, for example, protein kinase B
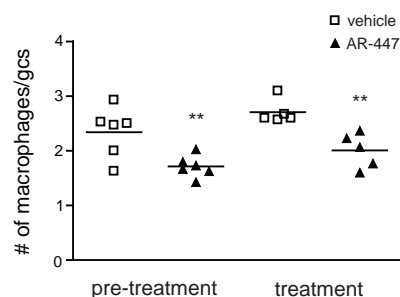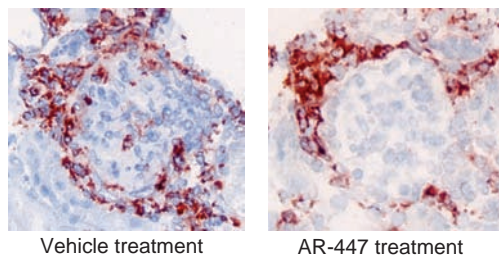
and JNK.[18 19] Our finding that two other p38MAPK inhibitors (AR-447 and RWJ-67657) at more pharmacologically relevant concentrations markedly reduced anti-MPO/Pr3 IgG-induced neutrophil respiratory burst and degranulation supports the hypothesis that p38MAPK is involved in ANCA IgG-induced neutrophil activation.

Besides involvement of p38MAPK in ANCA IgG-induced neutrophil activation, we found a role for p38MAPK in LPS-induced glomerular endothelial cell activation in vitro. LPS activated p38MAPK in human glomerular endothelial cells and podocytes, which is consistent with a recent study by Psotka et al.[20] In that study, increased phosphorylation of p38MAPK in conjunction with a profound increase in secretion of the proinflammatory cytokines IL-6, IL-8 and MCP-1 was observed upon LPS treatment. Similar to our results, the authors found that the LPS-induced increase in cytokine production was larger in glomerular endothelial cells compared to podocytes. Increased levels of IL-6, IL-8 and MCP-1 have also been found in the plasma or urine of patients with ANCA-associated vasculitis.[21 22] Moreover, renal CXCL1, CXCL2 and MCP-1 mRNA levels were increased in the mouse model of ANCA-associated glomerulonephritis.[14] These observations suggest that IL-6, IL-8 and MCP-1 may be involved in ANCA-induced glomerular inflammation. In our
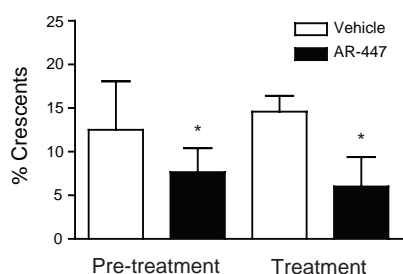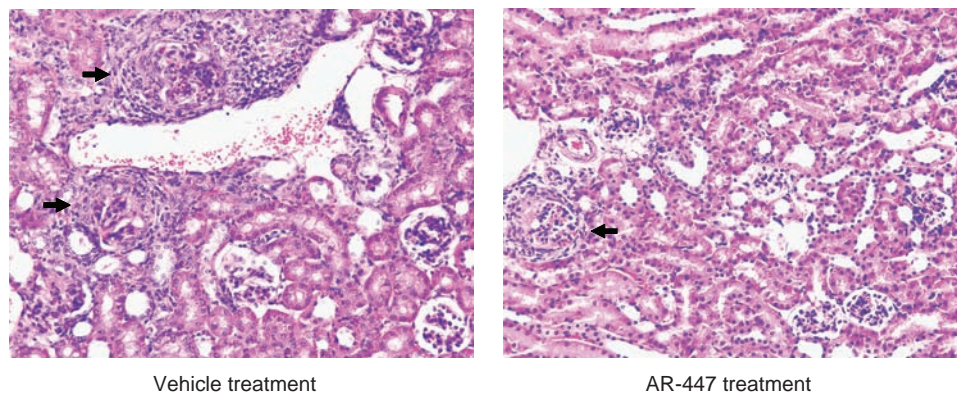
Figure 6 Inhibition of p38 mitogen-activated protein kinase (p38MAPK) reduces crescent formation in anti-myeloperoxidase (MPO) IgG/lipopolysaccharide (LPS)-induced glomerulonephritis in mice. A. Renal cryosections were immunohistochemically stained for neutrophils 1 day after glomerulonephritis induction in mice that were pretreated with AR-447 (30 mg/kg) or vehicle (n=6 per group). Representative images and quantification of glomerular neutrophils are shown. Original magnification 400×; Gcs, glomerular cross section. B. At 7 days after induction of glomerulonephritis, renal cryosections were stained for macrophages (n=5–6 per group). Representative images and quantification of glomerular macrophages are shown. Original magnification 200×. **p<0.01 compared to vehicle (pre)treated mice. C. The number of crescentic glomeruli was scored in kidney sections of mice that were pretreated or treated with AR-447 or vehicle 7 days after glomerulonephritis induction. Bars represent mean ± SD of 5–6 mice. *p<0.05 compared to vehicle (pre)treated mice.

study, we observed an LPS-induced increase in production of all cytokines in glomerular endothelial cells, whereas in podocytes only MCP-1 was increased. The increased MCP-1 expression was not dependent on p38MAPK. Previous studies in cultured serum-starved podocytes reported induction of MCP-1, IL-6

and granulocyte macrophage-colony stimulating factor upon LPS,[23 24] but involvement of p38MAPK was not investigated. In glomerular endothelial cells, we demonstrated that LPS-induced IL-6 and IL-8 production partially depends on p38MAPK. P38MAPK dependency for LPS-induced cytokine release has

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been shown for several other types of endothelial cells, including human umbilical vein,[25 26] human dermal microvascular,[27 28] human pulmonary artery[29] and rat pulmonary microvascular[30] endothelial cells. Our finding that LPS-induced cytokine production by glomerular endothelial cells was only moderately blocked upon p38MAPK inhibition may be due to involvement of other kinases and transcription factors, such as phosphoinositide 3 kinase (PI3K)[30 31] and nuclear factor κB.[30 32 33] Nevertheless, our results suggest that p38MAPK activation in glomerular endothelial cells can potentially contribute to glomerular inflammation during glomerulonephritis.

These in vitro observations prompted us to investigate the effects of p38MAPK inhibition on the development of anti-MPO IgG/LPS-induced glomerulonephritis in mice. In our experiments, a moderate reduction in early albuminuria and in the number of glomerular macrophages and crescentic glomeruli was demonstrated. This moderate reduction in renal injury upon p38MAPK inhibition in our model of anti-MPO IgG/LPS-induced glomerulonephritis is in contrast with the more prominent beneficial effects of P38MAPK inhibition observed in rat models of anti-glomerular basement membrane-induced glomerulonephritis.[34–37] These contrasting findings are not easily explained but may relate to differences in inflammatory response between the models or differences in specificity of the p38MAPK inhibitors used, that is, their ability to inhibit related kinases as well.

Our findings provoke the question why p38MAPK inhibition only partially reduces renal injury in anti-MPO IgG-induced glomerulonephritis in mice while it markedly reduces ANCA IgG-induced neutrophil activation in vitro. Several explanations are possible. First, ANCA-mediated neutrophil activation (eg, respiratory burst and degranulation) might not be important in the pathogenesis of ANCA-associated glomerulonephritis. A previous study demonstrated that ANCA-mediated endothelial injury is mediated by serine proteases released from ANCA-stimulated neutrophils rather than by superoxide, suggesting that the neutrophil respiratory burst is indeed less important for vasculitis development.[38] However, we demonstrate that AR-447 attenuates neutrophil respiratory burst and degranulation. Together with the fact that neutrophils are required for disease development,[39] it seems unlikely that neutrophil activation is not important for the development of ANCA-mediated vasculitis. Second, the development of inflammatory diseases, such as glomerulonephritis, is very complex. Other kinases involved in ANCA-induced neutrophil activation, for example, PI3K,[40 41] ERK[4 42] and the tyrosine kinase Syk,[43] may remain active to contribute to disease activity. This hypothesis is supported by recent results from several independent clinical trials, showing that pharmacological inhibition of p38MAPK with specific p38MAPK inhibitors in patients with arthritis caused only a moderate reduction in clinical symptoms and a transient reduction in inflammation markers.[44–46] Finally, p38MAPK might be involved in the process of neutrophil priming (that is, antigen translocation) rather than in ANCA-induced signalling. In contrast to the in vitro situation where only one proinflammatory stimulus (TNFα) is used for priming, multiple stimuli can contribute to neutrophil priming in vivo. Possibly, these additional stimuli induce p38MAPK-independent neutrophil priming in vivo. This would suggest that inhibition of a kinase that is specifically activated upon ANCA IgG would be more beneficial for the treatment of ANCA-mediated diseases. Interestingly, in ANCA-activated neutrophils activation of the γ isoform of PI3K (PI3Kγ) has been demonstrated, and its specific inhibition substantially attenuated ANCA-induced glomerulonephritis in

mice.[41] Taken together, these observations suggest that specific inhibition of p38MAPK ameliorates some aspects of the disease, yet does not fully counteract the inflammatory processes induced by ANCAs in vivo.

In conclusion, this study shows that inhibition of p38MAPK markedly reduces ANCA-induced neutrophil activation in vitro but only partially reduces pathogenicity in vivo when the drug was administered prior to disease induction and after disease onset. These data suggest that besides p38MAPK activity other signalling pathways contribute to the pathogenesis of ANCA-mediated disease.

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