Macrophage-Mediated Renal Injury Is Dependent on Signaling via the JNK Pathway

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Abstract. Macrophage accumulation is a prominent feature in most forms of human glomerulonephritis and correlates with renal dysfunction. Macrophages can directly mediate acute renal injury in animal models, but the mechanisms of macrophage activation required for mediating renal injury are unknown. This study examined whether activation of the Jun amino terminal kinase (JNK) signaling pathway is necessary for macrophage-mediated renal injury. An adoptive transfer model was used in which rats were immunized with sheep IgG (day −5), made leukopenic by administration of cyclophosphamide (CyPh) (day −2), and then injected with sheep anti-glomerular basement membrane (GBM) serum (day 0). Animals were then given an intravenous injection of bone marrow-derived macrophages (BMM) (day 1) and killed 24 h later (day 2). The induction of proteinuria and glomerular cell proliferation (PCNA+ cells) in CyPh-treated anti-GBM disease was dependent on transfer of BMM. Exposure of BMM to the specific JNK inhibitor, SP600125, for 3 h before adoptive transfer had no effect on glomerular accumulation of BMM in CyPh-treated anti-GBM disease. However, SP600125 treatment of BMM caused a 75% reduction in proteinuria and a 70% reduction in glomerular cell proliferation ($P < 0.01 \text{ versus} \text{ vehicle or untreated BMM}$). In conclusion, this study has defined a critical role for the JNK signaling pathway in macrophage-mediated renal injury.

Accumulation of macrophages within the kidney occurs in most types of primary and secondary human glomerulonephritis (1,2). The density of macrophage accumulation correlates with the degree of renal dysfunction and is predictive of disease progression (2–5), suggesting that macrophages mediate acute and progressive renal injury. Similarly, many temporal studies in both immune and non-immune–initiated models of kidney disease have shown a clear association between macrophage accumulation and the development of renal injury (reviewed by Nikolic-Paterson et al. [2]). However, not all studies have found concordance between increased macrophage numbers and more severe renal injury (6,7), suggesting that there may be significant functional heterogeneity within the infiltrating macrophage population. Indeed, macrophages can perform many different functions according to the local microenvironment in which they are present, ranging from a pro-inflammatory response to microbial infection to the removal of apoptotic cells, downregulation of the innate/immune response, and tissue repair. This is evident by the different states of macrophage activation seen in response to a diverse range of stimuli (8); whether macrophages induce injury or promote repair will depend on the nature of the stimuli present within the damaged kidney.

A variety of strategies have been used to examine the role of macrophages in experimental renal injury, including macrophage depletion (e.g., irradiation, cyclophosphamide, lipid encapsulated drugs) or blockade of molecules involved in monocyte recruitment into the inflamed kidney (e.g., chemokines, cytokines, and adhesion molecules) (reviewed by Nikolic-Paterson et al. [2]). In general, these studies support a role for macrophages in the induction and progression of experimental renal injury, although they all suffer from the limitation that the strategies employed affect more than just macrophages.

Demonstrating a role for macrophages in the resolution of renal injury has been more difficult. One approach to this question has been to manipulate the activation status of macrophage populations in vitro and then transfer these cells into animals (9). The transfer of NR8383 macrophages into normal rat glomeruli via renal artery perfusion induces a number of pro-inflammatory responses, which are not seen when the function of the transcription factor nuclear factor-$\kappa$B (NF-$\kappa$B) is inhibited in the transferred macrophages (10). In addition, the transfer of macrophages modified to secrete antiinflammatory cytokines (IL-1ra, IL-4, or IL-10) has been shown to suppress renal injury in animal models of anti-GBM disease (11–13). However, it is not clear whether this suppression of renal injury is due to modulation of macrophage function and/or effects of the secreted cytokines upon intrinsic renal cells and other leukocytes.

We are interested in the mechanisms of macrophage activation that lead to renal injury. To examine this, we have developed an acute model in which renal injury is dependent on
adoptive transfer of macrophages (14). In this model, the degree of proteinuria and glomerular cell proliferation is proportional to the number of transferred glomerular macrophages (14). We have demonstrated that activation by interferon-γ (IFN-γ) augments macrophage-mediated renal injury through a glucocorticoid-sensitive mechanism, although macrophage-mediated renal injury was not dependent on IFN-γ activation (15). The current study examined whether macrophage activation via the Jun amino terminal kinase (JNK) is critical for macrophage-mediated renal injury.

The JNK signaling pathway is a member of the stress-activated protein kinase (SAPK) family, which provide the link whereby cells can respond to extracellular (or intracellular) stress by changing which genes are switched on (i.e., actively transcribed). Exposure of cells to a wide range of stresses, such as endotoxin, reactive oxygen species, pro-inflammatory cytokines (IL-1, TNF-α), chemokines (MCP-1), ceramides, growth factors, irradiation, or osmotic stress induces a cascade of phosphorylation reactions involving specific cytoplasmic kinases which leads to phosphorylation, and thus activation, of JNK (16). The active JNK then enters the nucleus and phosphorylates a variety of transcription factors, including c-Jun which can then combine with c-fos to make the transcription factor, activator protein-1 (AP-1). This leads to a change in the pattern of gene transcription in the cell, such as switching on production of a number of pro-inflammatory mediators. The inflamed glomerulus produces cytokines (IL-1, TNF-α), chemokines (MCP-1), and reactive oxygen species, making the JNK signaling pathway a good candidate for inducing macrophage activation and renal injury.

The aim of the current study was to determine whether signaling through the JNK pathway is essential for macrophage-mediated renal injury. To study macrophages in isolation from other renal cells and inflammatory cells, we used an adoptive transfer model in which renal injury is dependent on transfer of cultured bone marrow-derived macrophages (BMM).

**Materials and Methods**

**Reagents**

The following monoclonal antibodies (mAb) were used in this study: ED1, anti-rat CD68, which labels monocytes and macrophages (17,18); OX-42, anti-rat CD11b, which labels monocytes, most macrophage populations, and some dendritic cells (19); PC-10, anti-proliferating cell nuclear antigen (PCNA), which labels cells in the G1, S, and G2 phase of the cell cycle (Dako Ltd, Glostrup, Denmark); 1A29, anti-rat CD54 (ICAM-1) (Serotec, Oxford, UK); WT.1, anti-rat CD11a (LFA-1) (Serotec); KM-1, anti-phospho c-Jun, which reacts with c-Jun phosphorylated at Serine 63 (Santa Cruz Biotechnology Inc, Santa Cruz, CA); anti-phospho p38, which reacts with the dual phosphorylated forms of all p38 isoforms (Sigma-Aldrich, St Louis, MO); anti-a1-tubulin (Sigma-Aldrich, Castle Hill, NSW, Australia); and 73.5, a mouse monoclonal antibody against human leukocytes that does not react with rat tissues was used as negative control.

Peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated mouse anti-peroxidase complexes (PAP) were purchased from Dako Ltd. FITC-conjugated sheep anti-mouse IgG F(ab’), fragment (AMRAD Biotec, Boronia, VIC, Australia) was used in flow cytometry. Lipopolysaccharide (LPS) prepared from *Escherichia coli* (L2630) was purchased from Sigma-Aldrich. The specific JNK inhibitor SP600125 (20,21) was a gift from Celgene Corporation (San Diego, CA). In kinase assays using purified proteins, SP600125 has been shown to specifically inhibit the activity of all 3 JNK isoforms but to lack inhibitory activity for a wide range of other enzymes (i.e., ERK2, p38β, IkB kinases 1 and 2, protein kinase A and C, Na/K ATPase, iNOS, phospholipase A2, NSF receptor tyrosine kinase, monoamine oxidase) (20,21). In addition, SP600125 has been shown to inhibit JNK activation in cell-based kinase assays over a concentration range of 1 to 50 µM, with an IC50 value between 5 and 10 µM, and the use of 25 µM SP600125 did not affect phosphorylation of extracellular-signal regulated kinase (ERK), p38, AT2, IκBα, or IκB kinase 1 (20).

**Bone Marrow-Derived Macrophages**

BMM were prepared by flushing the dissected femur and tibia of male inbred Sprague-Dawley rats using 0.2% EDTA in sterile phosphate-buffered saline (PBS). Cells were tease apart, washed, and resuspended in Dulbecco modified Eagle medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. The stromal cell population was removed by a 2-h adherence step in Petri dishes at 37°C, with the nonadherent fraction recovered and then cultured for 5 d in the presence of 20% L-cell conditioned medium as a source of macrophage colony-stimulating factor. After 5 d of culture, cells were at least 90% macrophages as judged by flow cytometry using CD68 (ED1) and CD11b (OX-42) monoclonal antibodies. All *in vitro* and *in vivo* experiments used BMM after 5 d of culture. For addition of SP600125 to BMM, the drug was first dissolved in the vehicle DMSO (Sigma-Aldrich) and then diluted in culture medium to a final concentration of 0.2% DMSO.

**Western Blot Analyses**

BMM were cultured (5 × 10⁶ cells) in 4-ml wells under various conditions, washed in PBS, and then lysed directly in 0.4 ml of SDS-PAGE sample buffer, left for 10 min; cell debris was then removed by centrifugation at 15000 × g for 20 min, and the supernatant was aliquoted and stored at −80°C. Lysates were run on 12.5% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA) by electroblotting in 25 mM Tris-HCl, pH 8.5, 192 mM NaCl, 0.05% Tween 80 with 10 or 20 µM SP600125 in 0.2% DMSO or vehicle alone, for 30 min before a 24-h stimulation with 100 ng/ml LPS, 10 ng/ml human IL-1β (PeproTech, London, UK), or 10 ng/ml mouse tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN). Secretion of Cytokine Production

BMM were seeded at 1 × 10⁶ cells in 1-ml wells and incubated with 10 or 20 µM SP600125 in 0.2% DMSO or vehicle alone, for 30 min before a 24-h stimulation with 100 ng/ml LPS, 10 ng/ml human IL-1β (PeproTech, London, UK), or 10 ng/ml mouse tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN). Secretion of
monocyte chemoattractant protein-1 (MCP-1) or TNF-α into the culture medium was measured by ELISA according to the manufacturer’s instructions (OptEIA; DB PharMingen, San Diego, CA). Assays were performed using 4 replica wells per sample.

**Analysis of Nitric Oxide Production**

BMM were seeded at 1 × 10^6 cells in 1 ml wells and incubated with different concentrations of SP600125 in 0.2% DMSO or vehicle alone for varying time periods before a 24-h stimulation with 100 ng/ml LPS. The accumulation of nitrite, a stable metabolite of nitric oxide, was measured in cell culture supernatants by the Greiss assay. Briefly, 50 μl of supernatant was combined with an equal volume of Greiss agent and incubated at room temperature for 10 min. The colored reaction product was measured by spectrophotometry at 550 nm with a reference calibration curve of known concentrations of sodium nitrite. Data are expressed as nanomoles of nitrite secreted by 10^6 cells in a 24-h period.

**Flow Cytometry**

BMM macrophages were incubated with 20 μM SP600125 in 0.2% DMSO or vehicle alone for 30 min before the addition of 100 ng/ml LPS (or medium alone) for 18 h. Cells were harvested using 0.02% EDTA/PBS and then resuspended in ice-cold PBS containing 1% FCS and 0.02% NaN₃. To detect adhesion molecule expression, cells were incubated with primary antibodies for 60 min, washed three times, and then incubated with FITC-conjugated sheep anti-mouse IgG diluted in 1% FCS, 10% heat-inactivated normal rat serum, 0.02% NaN₃, in PBS. The 73.5 MAb was used as an isotype-matched negative control. Cells were analyzed using a FACScan flow cytometer (Cytomation, Fort Collins, CO) equipped with Cyllops SUMMIT software.

**Adoptive Transfer Model of Macrophage-Mediated Renal Injury**

A modified version of rat anti-GBM disease was used as described previously (14). Inbred male Sprague-Dawley rats (body weight, 150 to 180 g) were obtained from Monash Animal Services. Rats were immunized by subcutaneous injection of 5 mg of sheep IgG in Freund’s complete adjuvant followed 5 d later by intravenous injection of sheep anti-rat GBM serum (day 0). Animals were made leukopenic (≥97% reduction in white blood cell numbers) by an intraperitoneal injection of 165 mg/kg cyclophosphamide (CyPh) given 2 d before administration of sheep anti-rat GBM serum. Transfer of BMM macrophages (6 × 10^7 cells per animal) was given by tail vein injection on day 1. Animals were placed in metabolic cages for a 24-h urine collection and then killed on day 2.

BMM were incubated with medium alone (Nil), medium containing 0.2% DMSO (Vehicle), or medium containing 20 μM SP600125 in 0.2% DMSO for 3 h and then washed twice before transfer into recipient animals. The same number of BMM (6 × 10^7 cells) was transferred into each animal irrespective of treatment. Washing of cells prevented carryover of SP600125 or vehicle into the recipient animals.

**Biochemical Analysis**

Protein excretion in 24-h urine collections was determined using the benzethonium chloride method. Whole blood cell counts were performed on a Cell-Dyn 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL) using heparinized blood collected from tail veins. All analyses were performed by the Department of Biochemistry, Monash Medical Centre.

**Immunohistochemistry**

ED1⁺ macrophages were detected in paraffin sections (4 μm) of methylcarnoy-fixed tissue using a three-layer peroxidase-based detection method, as described previously (22). Sections were dewaxed, hydrated in PBS, heated for 10 min in 0.1 M sodium citrate pH 6.0 in a microwave oven, washed in PBS, blocked with 10% FCS and 10% normal sheep serum in PBS for 30 min, drained, incubated with ED1 mAb in 10% normal rat serum, 1% BSA in PBS overnight at 4°C, washed in PBS, and then endogenous peroxidase–inactivated by incubation in 0.3% H₂O₂ in methanol. Sections then were washed in PBS, incubated sequentially with peroxidase-conjugated goat anti-mouse IgG and then mouse PAP complexes, and developed with 3,3-diaminobenzidine to produce a brown color. Immunostaining with the PC-10 (PCNA) antibody used the same protocol, except that the PAP complex amplification step was omitted.

The number of ED1⁺ macrophages or PCNA⁺ proliferating cells was counted in at least 50 glomerular cross-sections (gcs) per animal. All scoring was performed on blinded slides.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Comparison of three groups or more used one-way ANOVA, and individual group means were compared post-test with the Tukey Multiple Comparison Test. All values are expressed as mean ± SD.

**Results**

**SP600125 Inhibits JNK Activation in BMM**

Stimulation of BMM with a 10-min exposure to LPS induced activation of the JNK signaling pathway as demonstrated by phosphorylation of the JNK-specific target, c-Jun (Figure 1). Incubation of BMM with the specific JNK inhibitor, SP600125, caused a dose-dependent reduction in LPS-induced phosphorylation of c-Jun (Figure 1).

The effect of JNK inhibition on various parameters of macrophage activation was examined. Incubation of BMM with the optimal dose of 20 μM SP600125 had no effect on basal expression of the adhesion molecules CD11a (leukocyte function-associated antigen-1 [LFA-1]) or CD54 (intercellular adhesion molecule-1 [ICAM-1]). LPS-induced upregulation of CD11a expression was prevented by the addition of SP600125 (Figure 2a); however, LPS-induced upregulation of CD54 expression was not affected (Figure 2b). In a different assay of macrophage activation, SP600125 caused a profound inhibition of MCP-1 and TNF-α secretion by LPS-stimulated BMM (Figure 3, a and d). In addition, SP600125 abrogated MCP-1 secretion by IL-1β– and TNF-α–stimulated BMM (Figure 3, b and c). Furthermore, SP600125 caused a small but significant dose-dependent reduction in nitric oxide secretion by LPS-stimulated BMM (Figure 4a).

An important issue to consider for the adoptive transfer model of macrophage-mediated renal injury is whether the inhibitory effect of SP600125 on BMM activation is maintained after the cells have been washed and injected into recipient animals; the washing is an essential step to avoid systemic administration of the drug. This was examined by incubation of BMM with 20 μM SP600125 for 3, 6, or 12 h.
before the cells were washed twice and then stimulated with LPS for 24 h to measure nitric oxide secretion. As shown in Figure 4b, there was a significant reduction in nitric oxide secretion demonstrating a prolonged effect of SP600125 on BMM activation even after the cells had been washed, thereby indicating the potential usefulness of this approach for adoptive transfer studies. As a second measure to validate this approach, BMM were incubated with SP600125 for 3 h, washed twice, and then left for 1 or 21 h before examining LPS activation of the JNK pathway in terms of phosphorylation of c-Jun. As shown in Figure 5a, there was a profound inhibition of JNK signaling 1 h after washing SP600125-treated BMM. Furthermore, a substantial reduction (>50%) in JNK signaling was still evident 21 h after washing SP600125-treated BMM (Figure 5b and c). As a specificity control, SP600125 was shown to have no effect upon LPS activation of the closely related p38 mitogen-activated protein kinase (MAPK) pathway (Figure 5b and c). In addition, incubation of BMM with 20 μM SP600125 or 0.2% DMSO vehicle for 24 h had no effect on cell viability as assessed by flow cytometry using annexin V staining (data not shown).

Adoptive Transfer of Macrophage Mediated in Rat Anti-GBM Disease

The injection of sheep anti-GBM serum into primed rats resulted in proteinuria (140.4 ± 56.5 versus 4.5 ± 0.9 mg/24 h in normal rats; P < 0.001) and glomerular cell proliferation (9.4 ± 0.7 versus 0.2 ± 0.1 PCNA+ cells/gcs in normal rats; P < 0.001). We have previously demonstrated that most proliferating glomerular cells at this stage of the disease are mesangial cells (14,15). These two parameters of renal injury were associated with significant glomerular macrophage accumulation (12.1 ± 1.9 versus 0.4 ± 0.1 ED1+ cells/gcs in normal rats; P < 0.001). Mild glomerular histologic damage was evident, with PAS deposits within the tuft, and capillary thrombi in small numbers of glomeruli (not shown). The mild nature of the glomerular lesions at this early stage of the disease was expected since the anti-GBM serum used in this model has very low endotoxin levels (23,24).

To examine renal injury mediated by transferred macrophages, it is necessary to remove circulating leukocytes in the recipient animal. This was done by administration of a single, large dose of CyPh 2 d before injection of anti-GBM serum. CyPh treatment caused a profound depletion (≥97% reduction) of circulating leukocytes. This abolished recruitment of circulating monocytes to the glomerulus (0.52 ± 0.25 ED1+ cells/
and prevented the development of proteinuria (5.9 ± 1.8 mg/24 h) and glomerular cell proliferation (0.55 ± 0.22 PCNA+ cells/glomeruli) (all \( P < 0.001 \) versus anti-GBM disease and not significantly different versus normal rats).

Adoptive transfer of BMM into the tail vein of rats with CyPh-treated anti-GBM disease led to mild glomerular histologic damage in terms of increased PAS-stained material within the tuft and capillary thrombi in small numbers of glomeruli (not shown). Glomerular accumulation of the BMM was confirmed by immunohistochemistry 24 h after transfer (Figure 6). Quantification of ED1+ cells demonstrated significant glomerular accumulation of macrophages 24 h after transfer (Figure 7a). Adoptive transfer of BMM into CyPh treated anti-GBM disease resulted in significant proteinuria and glomerular cell proliferation (Figures 6 and 7). No glomerular macrophage accumulation or renal injury is seen when transferring BMM via the tail vein into normal rats (14).

**SP600125 Inhibits Macrophage-Mediated Renal Injury**

Incubation of BMM with 20 \( \mu \)M SP600125 before adoptive transfer into CyPh-treated anti-GBM disease rats had no effect upon glomerular accumulation of the transferred cells (Figures 6c and 7a). However, SP600125 treatment resulted in a 75% reduction in proteinuria (Figure 7b) and a 70% reduction in glomerular cell proliferation (Figures 6f and 7c) compared with BMM treated with vehicle alone or untreated (Nil) BMM. Expression of the data as the degree of proteinuria or number of proliferating cells per glomerular ED1+ macrophage shows
that SP600125 treatment significantly inhibited the ability of transferred BMM to induce proteinuria and glomerular cell proliferation (Figure 8).

**Discussion**

Recent studies using adoptive transfer have demonstrated that macrophages can mediate acute renal injury (14). One of the strengths of the adoptive transfer approach is that it enables direct investigation of the mechanisms by which macrophages enter the kidney and mediate injury.

The present study examined the role of the JNK signaling pathway in macrophage-mediated renal injury. Treatment of BMM with the specific JNK inhibitor, SP600125, did not affect the ability of BMM, administered via the tail vein, to be recruited to the inflamed glomerulus. This was somewhat unexpected in view of our finding that SP600125 treatment prevented LPS induced upregulation of CD11a (LFA-1) on the surface of BMM. The CD11a adhesion molecule has been implicated in glomerular leukocyte recruitment in rat anti-GBM disease (25,26). It may be that CD11a is not involved in glomerular macrophage recruitment or that basal levels of CD11a expression are sufficient to enable efficient recruit-
In this model, CyPh treatment does not prevent upregulation of glomerular expression of leukocyte adhesion molecules (ICAM-1 and VCAM-1) or upregulation of chemokines (MCP-1) (14). Therefore, in the presence of glomerular inflammatory signals, we can conclude that signaling through the JNK pathway is not required for the recruitment of blood monocyte/macrophages into the glomerulus. However, this finding does not rule out the possibility that signaling via the JNK pathway is required for the induction of glomerular inflammation in response to deposition of anti-GBM antibodies.

In contrast to the lack of effect on the recruitment of BMM to the inflamed glomerulus, the substantial inhibition of renal injury seen with SP600125-treated cells demonstrates that JNK signaling is critical in macrophage-mediated renal injury. The first issue to consider is what stimuli might induce activation of the JNK pathway in macrophages entering the microenvironment of the inflamed glomerulus? A number of factors known to be present in the inflamed glomerulus in vivo have been shown to induce JNK signaling in macrophages in vitro. The pro-inflammatory cytokines TNF-α and IL-1 can induce JNK activation in macrophages (27). Hydrogen peroxide produced by exogenous or endogenous respiratory burst activity can induce JNK signaling in macrophages (28,29). Cross-linking of the Fc receptor on mouse BMM induces activation of the JNK pathway in association with TNF-α production (30). Bacterial

Figure 7. JNK blockade inhibits macrophage-mediated renal injury. Groups of six rats with CyPh-treated anti-GBM disease were given untreated BMM (BMM Nil), 0.2% DMSO vehicle–treated BMM (BMM Veh), 20 μM SP600125-treated BMM (BMM SP600), or no macrophage transfer (CyPh Ctl). Analysis was performed 24 h after BMM transfer. (a) The number of ED1+ macrophages per glomerular cross-section (gcs) was determined by quantification of immunostained sections. (b) Proteinuria was measured in 24-h urine collections. (c) The number of PCNA+ macrophages per gcs was determined by quantification of immunostained sections. Statistical analysis performed by ANOVA with Tukey Multiple Comparison post-test.

Figure 8. JNK blockade modulates the degree of renal injury induced by transferred macrophages. Groups of six rats with CyPh-treated anti-GBM disease were given untreated BMM (BMM Nil), 0.2% DMSO vehicle–treated BMM (BMM Veh), or 20 μM SP600125–treated BMM (BMM SP600), as shown in Figure 6. The (a) degree of proteinuria or (b) the number of glomerular PCNA+ proliferating cells is expressed as a ratio to the number of ED1+ macrophages per gcs for each animal. Bar represents the mean. Statistical analysis performed by ANOVA with Tukey Multiple Comparison post-test.
endotoxin is a potent activator of the JNK pathway, which was confirmed in the current study. In addition, active components of the complement cascade can induce JNK signaling in peripheral blood monocytes (31).

A second question is what are the JNK-dependent mechanisms responsible for macrophage-mediated renal injury? We currently have no definitive information on this question. Indeed, we do not know exactly how macrophages cause proteinuria or mesangial cell proliferation in this model of acute renal inflammation. There are many macrophage products with the potential to cause renal injury, including pro-inflammatory cytokines (IL-1, TNF-α, macrophage migration inhibitory factor), growth factors (platelet-derived growth factor, fibroblast growth factor), nitric oxide, reactive oxygen species, eicosanoids, and metalloproteinases (MMP) (reviewed by Nikolic-Paterson et al. [2]). The production of some of these mediators in macrophages, or other cell types, is partially dependent on JNK signaling. We demonstrated that the production of nitric oxide secretion is partially dependent on JNK signaling. This is consistent with previous studies showing that JNK signaling plays a significant role in IFN-γ plus LPS or IFN-γ plus TNF-α-induced iNOS mRNA synthesis and nitric oxide secretion in mouse BMM and macrophage cell lines (32,33), although it should be noted that signaling through other pathways, such as ERK, have also been shown to play a role in macrophage nitric oxide production (34). Monocyte production of TNF-α in response to stimulation with LPS or MCP-1 operates in a JNK-dependent fashion (35,36); in the present study, BMM production of MCP-1 and TNF-α (factors known to promote renal injury) was shown to be particularly sensitive to JNK inhibition. In addition, studies in non-macrophage cell types have demonstrated a role for the JNK signaling pathway in the production of cyclooxygenase-2, and MMP1, 9, and 13 (20,37–39).

Blockade of JNK signaling substantially reduced but did not abrogate macrophage-mediated renal injury. This could be due to an incomplete inhibition of JNK signaling, although significant inhibition of JNK signaling was still evident 21 h after washing SP600125-treated BMM in vitro. Alternatively, the inability of JNK blockade to completely inhibit macrophage-mediated renal injury may indicate that other pathways of macrophage activation are involved in this process. Other candidate activation pathways for macrophage-mediated renal injury include p38 MAPK and ERK, which play important roles in innate and immune responses (40). Another important pathway of macrophage activation is NF-κB. Inhibition of NF-κB activity in macrophages transferred into normal glomeruli via renal artery perfusion prevents the induction of the pro-inflammatory response seen with transfer of control macrophages (10). In addition, blockade of glomerular NF-κB signaling via a gene therapy approach has been shown to suppress macrophage accumulation and renal injury in rat anti-GBM disease (41). Of note, the concentration of SP600125 used to treat BMM has no effect on p38, ERK, or NF-κB signaling pathways in cell-based kinase assays (20).

The dependence of macrophage-mediated renal injury on JNK signaling contrasts with macrophage activation by IFN-γ. We have previously shown that IFN-γ activation is not necessary for macrophage-mediated renal injury, but that it can augment macrophage-mediated renal injury (15). A potential interplay between the two mechanisms is suggested by studies in which IFN-γ augmented Fc receptor-mediated JNK signaling in mouse BMM (42).

There are limitations inherent with using drug-based approaches to examine the role of a specific molecular pathway in vivo. The usual problems of drug delivery, blood and tissue concentrations, and drug half-life have been circumvented in the present study by adding SP600125 to cultured BMM at a known concentration before these cells then were washed and transferred into recipient animals. In our studies, we used SP600125 at a dose that has been shown to specifically inhibit JNK activity in cultured cells (20), and we verified that SP600125 did not affect LPS-induced p38 activation in BMM. However, testing the specificity of any drug is limited to a relatively small number of possible molecular targets; therefore, it cannot be formally excluded that SP600125 affects some JNK independent function relevant to macrophage-mediated renal injury.

In summary, the current study has demonstrated a critical role for the JNK signaling pathway in macrophage-mediated renal injury and suggests that targeting this pathway may have therapeutic potential in rapidly progressive glomerulonephritis.

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