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Antibody blockade of TNF-α reduces inflammation and scarring in experimental crescentic glomerulonephritis

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Antibody blockade of TNF-α reduces inflammation and scarring in experimental crescentic glomerulonephritis.

Background. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine produced by macrophages, and by renal mesangial and tubular epithelial cells. It stimulates the release of interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor-β (TGF-β). Blockade of TNF-α is currently used clinically in several autoimmune inflammatory diseases. We hypothesised that blocking TNF-α with a monoclonal antibody would prevent inflammation and renal fibrosis in crescentic glomerulonephritis.

Methods. Nephrotoxic nephritis was induced in Wistar Kyoto (WKY) rats by intravenous injection of rabbit antirat glomerular basement membrane (GBM) nephrotoxic serum (NTS). Anti-TNF-α monoclonal antibody or saline was given intraperitoneally three times per week in four protocols: experiment 1, days 0 to 7; experiment 2, days 0 to 14 and days 4 to 14; experiment 3, days 4 to 28; and experiment 4, days 14 to 28.

Results. In experiment 1, rats treated from disease induction had less glomerular fibrinoid necrosis and fewer glomerular macrophages at day 7. In experiment 2, rats treated from day 0 or day 4 showed improved renal function, as judged by serum creatinine, with a significant reduction in crescents. In experiment 3, anti-TNF-α treatment significantly reduced urine protein to creatinine ratio and urinary MCP-1 levels. Serum creatinine was preserved at both day 14 and day 28. Tubulointerstitial inflammation, glomerular and tubulointerstitial scarring, and markers of fibrosis [α-smooth muscle actin (α-SMA) and type IV collagen] were significantly less in treated rats at day 28. In experiment 4, serum creatinine was higher and tubulointerstitial scarring was less in delayed-treated animals.

Conclusion. Neutralization of endogenous TNF-α reduces glomerular inflammation, crescent formation, and tubulointerstitial scarring, with preservation of renal function, in experimental crescentic glomerulonephritis. TNF-α blockade is effective even when introduced at the time of maximum glomerular inflammation.

Key words: crescentic glomerulonephritis, glomerular basement membrane (GBM), nephrotoxic nephritis, tumour necrosis factor-α (TNF-α), fibrosis, Wistar Kyoto (WKY) rat.

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Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine and mediator of many immune functions [1], which is produced by a wide variety of cells, particularly monocytes, activated macrophages, and fibroblasts. In the kidney it may be produced by intrinsic renal cells, including mesangial cells and tubular epithelial cells, in response to proinflammatory stimuli such as platelet-derived growth factor (PDGF) and TNF-α itself. TNF-α is present in a soluble form (17 kD), to which most of the biologic effects are attributable, and also a membrane-bound form (26 kD).

The TNF ligand receptor interaction is mediated via two distinct cell surface receptors: TNFR1 (p55), which predominates, and TNFR2 (p75). Both receptors act synergistically for cell proliferation and maturation, cytotoxicity, and antiviral activity. TNF-α ligation to TNFR1 and TNFR2 can activate caspase 8 via intracellular signaling events involving death domains, or by activating c-Jun NH2-terminal kinase (JNK) which is involved in phosphorylating the transcription factor c-jun, promoting apoptosis [2]. Alternatively, a TNF-induced survival signal can trigger a cascade of intracellular events leading to the activation of the transcription factor nuclear factor-κB (NF-κB), which is involved in the suppression of apoptosis and initiates gene transcription of many molecules involved in acute and chronic inflammatory responses [3]. These molecules include proinflammatory cytokines, chemokines, and growth factors, such as interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β) and macrophage migration inhibitory factor (MIF), which act to amplify the inflammatory response. TNF blockade can inhibit NF-κB activation, promoting increased apoptosis of inflammatory cells, and reducing the inflammatory response. Cell-type specific regulation can determine which

signaling pathway and transcription factors are recruited to the TNF promoter. TNF-α can also be immunostimulatory, by inducing expression of major histocompatability complex (MHC) class I and II molecules, and of adhesion molecules [e.g., intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) on antigen-presenting cells (APCs).

Overproduction of TNF-α plays a key role in acute and chronic inflammatory diseases. It has been implicated in the pathogenesis of several T-cell–mediated autoimmune diseases, including multiple sclerosis [4], rheumatoid arthritis [5], and inflammatory bowel disease [6]. Inhibition of endogenous TNF with monoclonal antibodies or soluble TNF receptors interferes with the action of the cytokine. This approach is currently used to treat inflammatory diseases such as Crohn's disease and rheumatoid arthritis [7–9], and has been successful in modulating disease progression.

There is considerable evidence that TNF-α plays a role in glomerular inflammation and scarring [10]. In an animal model of minimal change nephropathy, TNF-α was produced within glomeruli by mesangial cells, epithelial cells, and infiltrating macrophages and monocytes [10, 11]. Systemic administration of TNF-α caused glomerular damage in normal rabbits [12], and increased the degree of glomerular injury in rats with nephrotoxic nephritis [12, 13].

Neutralization of endogenous TNF-α in rats with crescentic nephritis using soluble TNFR p55, a soluble truncated fragment of the extracellular domain of TNFR1, prevented acute glomerular inflammation and crescent formation [14, 15]. In a rat model of experimental crescentic nephritis, MIF was up-regulated in renal intrinsic cells and infiltrating macrophages. When treated with TNF binding protein, a dimeric form of the soluble receptor, serum levels of MIF decreased, along with a reduction in renal injury and interstitial fibrosis, indicating that TNF-α induces MIF expression and that together they enhance renal inflammation [16].

Experiments using TNF-α−/− knockout mice have also demonstrated a reduction in severity of crescentic nephritis induced by antiglomerular basement antibody [17]. These TNF-α−/− animals have also been used to create chimeric mice, to distinguish the role of bone marrow–derived versus intrinsic renal cell TNF-α [18]. These studies suggest that intrinsic renal cells are the major contributor to TNF-mediated injury.

The role of TNF-α in the pathophysiology of renal fibrosis has been examined in a mouse model of unilateral ureteric obstruction (UUO) [19, 20] in which individual TNF-α receptors, TNFR1 or TNFR2, were knocked out. Mice deficient in either receptor, especially TNFR1, demonstrated reductions in interstitial volume, myofibroblast differentiation, collagen deposition, and NF-κB activation, compared to the wild-type.

The only previous study using anti-TNF-α antibodies in a rat model of nephrotoxic nephritis was a 24-hour experiment, using a polyclonal anti-TNF antibody, which reduced proteinuria and glomerular accumulation of neutrophils [21]. We hypothesized that blocking TNF-α with a monoclonal antibody, at either acute or chronic stages of disease, would be effective in preventing and treating experimental crescentic glomerulonephritis. The model we have used is induced in Wistar Kyoto (WKY) rats by a single intravenous injection of rabbit antirat glomerular basement membrane (GBM) serum. This initiates a vigorous inflammatory response, characterized by up-regulation of proinflammatory cytokines and adhesion molecules, together with recruitment of leukocytes into the glomeruli. The cell influx is underway by day 1 of disease and is predominantly monocytic. Glomerular leukocyte counts peak at day 3 or 4. Glomerular fibrinoid necrosis and crescent formation begin by day 7, and peak around day 14. Tubulointerstitial inflammation accompanies crescent formation and is followed by increasing interstitial fibrosis, tubular damage and progressive glomerulosclerosis [22]. Creatinine clearance starts to fall and serum creatinine starts to rise by day 14 of nephrotoxic nephritis. Animals develop end-stage renal failure by week 5 or 6.

In the present study we have examined the effect of a blocking antibody to TNF-α, administered either during the phase of acute inflammation or during the scarring phase of nephrotoxic nephritis. Our results clearly show that inhibition of TNF-α reduces both acute glomerular inflammation and glomerular and interstitial fibrosis, and prevents the development of renal failure.

METHODS

Animals

Male WKY rats aged between 6 and 8 weeks (weight 200 to 250 g) were purchased from Charles River Laboratories (Margate, UK) and had free access to standard laboratory diet and water. All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Antibodies

A blocking IgG2a monoclonal antibody to rat TNF-α (C432) (Centocor, Philadelphia, PA, USA) was used in all experiments. In prior pilot experiments and in our previous studies [14, 23, 24], an isotype matched control monoclonal antibody showed no effect on renal injury in comparison to saline, and therefore saline injections were used as a control in the present study.

Nephrotoxic nephritis

Nephrotoxic serum was prepared in rabbits by standard methods [25]. Briefly, rat glomeruli were isolated, sonicated, and lyophilized to provide a rat GBM preparation with <5% tubular contamination. Rabbits were immunized with rat GBM in Complete Freund's Adjuvant (CFA), then boosted at monthly intervals with GBM in Incomplete Freund's Adjuvant (IFA), until high titer anti-GBM antiserum was obtained at test bleeds. Nephrotoxic nephritis was induced in male WKY rats by intravenous injection of 0.1 mL of nephrotoxic serum, as previously described [22].

Assessment of renal disease

Urine was collected by placing rats into metabolic cages for 24 hours, at weekly intervals, with free access to food and water. Proteinuria was determined by the sulphosalicylic acid method [26]. Tail artery bleeds were performed at weekly intervals, under light anesthesia using isoflurane, to allow determination of serum creatinine using an Olympus AU600 analyser (Olympus, Eastleigh, UK). Urinary creatinine concentration was measured to permit calculation of urine protein to creatinine ratio. Rats were killed under isoflurane anesthesia, and blood was collected from the abdominal aorta.

Renal histology

At sacrifice, one portion of kidney was fixed in 10% formal saline and embedded in paraffin. Kidney tissue was also snap frozen in isopentane, immersed in liquid nitrogen, and stored at −70°C. Paraffin sections were stained with hematoxylin and eosin, and periodic acid-Schiff (PAS), and examined in a blinded fashion for glomerular scarring, tubular damage, and interstitial scarring. The percentage of glomeruli containing crescents was determined by examining 100 consecutive glomeruli per section. Glomerular scarring at day 28 was assessed in hematoxylin and eosin sections by scoring the number of glomerular quadrants containing scars.

Immunohistochemistry and immunofluorescence

The monoclonal antibodies used were specific for rat monocytes/macrophages (ED1) (Serotec, Oxford, UK); CD8 cells (OX8) (Serotec); ED(A) fibronectin [ED(A)FN] (IST-9) (Harlan Sera-Lab, Loughborough, UK); type IV collagen (Southern Biotechnology, Birmingham, AL, USA); inducible nitric oxide synthase (iNOS) (clone 6) (BD Transduction Laboratories, Oxford, UK); α-smooth muscle actin (α-SMA) (IA4); goat antirabbit IgG fluorescein isothiocyanate (FITC); and rabbit antirat IgG FITC (Sigma-Aldrich, Poole, UK).

For direct immunofluorescence for rabbit and rat IgG, frozen sections were fixed in acetone and blocked with 20% normal goat or rabbit serum, respectively, then incubated for 2 hours with FITC-conjugated monoclonal antibody to rabbit IgG (1:80) or rat IgG (1:20). For ED(A)FN staining, frozen sections were fixed in acetone, and endogenous peroxidase blocked using 0.03% hydrogen peroxide in methanol. Sections were blocked with 20% normal rabbit serum and incubated overnight at 4°C with monoclonal antibody to ED(A)FN (1:1000). Sections were washed and incubated with a rabbit antimouse biotinylated secondary antibody (Dako, Glostrup, Denmark). Bound antibody was detected using the avidin-biotin complex (ABC) method (Dako), with the chromogenic substrate 3,3′-diaminobenzidine (DAB) (Dako). Sections were counterstained with hematoxylin and mounted.

The other antibodies were used on paraffin sections at the following dilutions: ED1, 1:500; OX8, 1:200; iNOS, 1:50; α-SMA, 1:1000; and type IV collagen, 1:1000. Antigen retrieval was carried out by heating paraffin-embedded sections in 10% citrate buffer in a microwave oven, three times, each for 5 minutes. Sections were then treated as for frozen tissue, with blocking of endogenous peroxidase, blocking with 20% normal rabbit serum, and incubation overnight with primary monoclonal antibody at 4°C. Sections were washed and then incubated with biotinylated rabbit antimouse antibody for ED1, OX8, and α-SMA, or biotinylated rabbit antigoat antibody for type IV collagen. Bound antibody was detected using ABC followed by DAB. Sections were counterstained with hematoxylin and mounted. ED1-, OX8-, and iNOS-positive cells were counted in 25 consecutive glomeruli or interstitial areas in randomized sections.

Computer-aided image analysis of immunofluorescence and immunohistochemistry

To quantify glomerular and interstitial fibrosis, immunoperoxidase and immunofluorescence kidney sections were examined under ×20 magnification, using an Olympus BX40 microscope (Olympus Optical, London, UK) mounted with a Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, UK). Digital images were captured and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA), and color segmentation was performed for each slide individually, defining pixels that contained appropriate coloration. For each slide, 20 consecutive glomeruli or cortical areas were defined as an "area of interest," and the percentage of each cross-sectional area stained with the defined color was calculated. The final value for each slide was derived as the mean percentage area stained from 20 areas.

Circulating rat antirabbit Ig measurement

A direct enzyme-linked immunosorbent assay (ELISA) was performed to quantify the levels of circulating rat antirabbit Ig levels in serum samples. A 96-well ELISA plate was coated with rabbit IgG in carbonate buffer antibody (Sigma-Aldrich) at 100 μg/mL, at 4°C overnight. The plate was blocked with a blocking buffer [phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.1% Tween 20] for 1 hour at 37°C. Serum samples from experimental rats were diluted 1:200 in PBS, 1% BSA, and 0.1% Tween 20 and were incubated for 1 hour at 37°C. The detection antibody, alkaline phosphatase-conjugated rabbit antirat Ig (Sigma-Aldrich) diluted 1:1000, was added to wells and incubated for 1 hour at 37°C. The substrate p-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich) dissolved in carbonate buffer was added to the plate then incubated for 30 minutes at room temperature. The plate was read at 450 nm. Amounts of circulating rat antirabbit Ig were expressed as optical density (OD) units.

Urinary and serum MCP-1 measurement

A direct ELISA was performed to quantify the amount of MCP-1 in urine and serum samples. A 96-well ELISA plate was coated with a capture antibody, antirat MCP-1 (C4) (BD Pharmingen, Oxford, UK) at 1 μg/mL, at room temperature overnight. The plate was blocked with a blocking buffer (PBS, 1% BSA, 5% sucrose, and 0.05% NaN_3) for 1 hour at room temperature. Recombinant rat MCP-1 standards (Peprotech EC Ltd., London, UK) were serially diluted from 1 μg/mL to 2 ng/mL, and experimental urine (1:20) and serum (1:50) samples were incubated for 2 hours at room temperature. The detection antibody, biotinylated antirat MCP-1 (B4), (Peprotech EC Ltd.) at 0.3 μg/mL, was added to wells and incubated for 2 hours at room temperature. Streptavidin horseradish peroxidase (HRP) (Zymed, San Francisco, CA, USA) diluted 1:10,000, was then incubated for 20 minutes, followed by substrate reagent (R&D Systems, Minneapolis, MN, USA) for 20 minutes. One mole per liter H_2SO_4 was used to stop the reaction and the plate was read at 450 nm. Amounts of MCP-1 were expressed as ng/mmol creatinine.

Experimental design

Experiment 1. This was designed to determine an effective dose of anti-TNF-α monoclonal antibody for prevention of inflammation at an early stage of nephrotoxic nephritis, from day 0 to day 7. The first dose was administered 2 hours before the induction of disease. Nephrotoxic nephritis was induced in 12 WKY rats. Four were treated with anti-TNF-α monoclonal antibody, 1 mg/rat intraperitoneally on alternate days; four were treated with 4 mg/rat; and four received normal saline as a control. All rats were killed at day 7.

Experiment 2. This was designed to study the preventive and therapeutic effects of anti-TNF-α monoclonal antibody with continued treatment between days 0 and 14. Treatment was started either at day 0 (experiment 2a) or at day 4 (experiment 2b), by which time there is established glomerular inflammation. Nephrotoxic nephritis was induced in 18 WKY rats. Six rats were treated with anti-TNF-α monoclonal antibody, 4 mg/rat intraperitoneally on alternate days from day 0; six rats were treated with anti-TNF-α monoclonal antibody, 4 mg/rat intraperitoneally on alternate days from day 4; and six received normal saline. All rats were killed at day 14.

Experiment 3. This was designed to study the prolonged therapeutic effect of anti-TNF-α monoclonal antibody on renal function, and glomerular and interstitial scarring, with treatment from day 4 to day 28. Nephrotoxic nephritis was induced in 12 WKY rats. At day 4, six rats were treated with anti-TNF-α monoclonal antibody, 4 mg/rat intraperitoneally on alternate days; and six received normal saline. All rats were killed at day 28.

Experiment 4. This was designed to study the effect of late treatment with anti-TNF-α monoclonal antibody, started on day 14 when there is severe glomerulonephritis with maximal crescent formation. Nephrotoxic nephritis was induced in 10 rats. At day 14, five rats were treated with anti-TNF-α monoclonal antibody, 4 mg/rat intraperitoneally on alternate days; and five received normal saline. All rats were killed at day 28.

Statistics

Data are expressed as mean ± SEM. Repeated measure comparisons between test and control groups for in vivo blocking monoclonal antiboy experiments were by a two-way analysis of variance (ANOVA) test. Differences were considered significant if $P < 0.05$. Statistical calculations were performed using Prism software (GraphPad Prism Software Inc., San Diego, CA, USA).

RESULTS

Effect of anti-TNF-α monoclonal antibody in acute nephrotoxic nephritis

In experiment 1, rats were treated from day 0 to day 7 with anti-TNF-α monoclonal antibody at 1 mg/rat or 4 mg/rat on alternate days, to determine an effective dose for prevention. Rats treated with either 1 mg or 4 mg of anti-TNF-α monoclonal antibody showed reduced glomerular fibrinoid necrosis compared to control rats (1 mg 1.33 ± 0.18; 4 mg 0.89 ± 0.08; and control 1.58 ± 0.09 glomerular fibrinoid necrosis score), but this only reached significance at the higher dose (4 mg dose) ($P < 0.05$). There were fewer glomerular

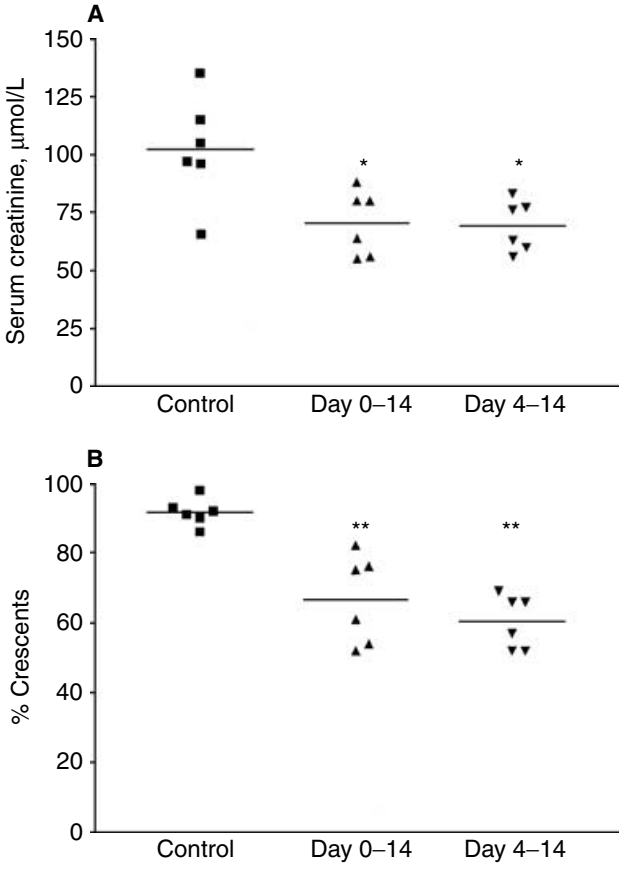

Fig. 1. Experiment 2. Renal function and percentage crescents in individual rats treated with anti-tumor necrosis factor-α (TNF-α) monoclonal antibody from day 0 or day 4 until day 14. (*A*) Serum creatinine was lower in animals treated from either day 0 (*$P < 0.05$) or from day 4 (*$P < 0.05$) compared with controls. (*B*) Percentage of crescents was lower in animals treated from either day 0 (**$P < 0.01$) or day 4 (**$P < 0.01$) compared with controls. Treatment began at day 0 (▲), day 4 (▼), and control (■).

macrophages in both treated groups than in the controls (1 mg 13.8 ± 0.7; 4 mg 13.5 ± 1.4; and control 23.5 ± 1.8 macrophages/glomerulus) ($P < 0.05$). There was no effect on the number of infiltrating CD8-positive cells (1 mg 2.2 ± 0.33; 4 mg 1.8 ± 0.56; and control 1.3 ± 0.61 CD8+ cells/glomerulus), urine protein to creatinine ratio (1 mg 0.95 ± 0.37; 4 mg 0.97 ± 0.24; and control, 0.98 ± 0.35 mg/μmol), or serum creatinine (1 mg 48.5 ± 3.2; 4 mg 44.5 ± 1.0; and control 53.8 ± 2.7 μmol/L). The dose of 4 mg/rat on alternate days was used in subsequent experiments.

In experiment 2, rats were treated from day 0 to day 14 or from day 4 to day 14 at the established dose of 4 mg/rat on alternate days. Renal function, as judged by serum creatinine, was better in rats treated with anti-TNF-α monoclonal antibody from the time of induction of disease (70.5 ± 5.7 μmol/L) ($P < 0.05$) or from day 4 (69.2 ± 4.5 μmol/L) ($P < 0.05$) compared with controls (102.3 ± 9.4 μmol/L) at day 14 (Fig. 1A). There was, however, no significant difference in urine protein to creatinine ratio at day 14, between animals treated from day 0 (0.95 ± 0.10 mg/μmol) or from day 4 (1.09 ± 0.10 mg/μmol) compared with controls (1.36 ± 0.26 mg/μmol). At day 14 in this model, around 90% of glomeruli contained cellular crescents [22]. There was a significant reduction in crescents ($P < 0.01$) in animals treated from either day 0 or day 4 (days 0 to 14 66.7 ± 5.2%; days 4 to 14 60.3 ± 3.1%; and control 91.6 ± 2.0%), (Fig. 1B). Cellular infiltration of macrophages (day 0 27.3 ± 2.5; day 4 26.2 ± 3.5; and control 24.2 ± 3.8 macrophages/glomerulus) and CD8-positive cells (day 0 0.85 ± 0.3; day 4 1.19 ± 0.3; and control 0.77 ± 0.2 CD8-positive cells/glomerulus) into the glomeruli was not different between groups. However, the number of iNOS-positive cells was increased in rats treated from day 0 and from day 4 compared to controls: day 0 6.2 ± 1.2 ($P< 0.05$); day 4 4.3 ± 1.4 (P = NS); and control 1.0 ± 0.32 iNOS-positive cells/glomerulus.

Effect of anti-TNF-α monoclonal antibody on progression of glomerulonephritis

In experiment 3, rats were treated from days 4 to 28. Assessment of urine protein to creatinine ratio demonstrated a reduction in the treated group at day 14 (treated 1.30 ± 0.23 and control 4.19 ± 0.69 mg/μmol) ($P < 0.01$), but not at day 28 (treated 1.11 ± 0.14 and control 1.48 ± 0.19 mg/μmol) (Fig. 2A). Serum creatinine at both day 14 (treated 58.2 ± 3.8 μmol/L and control 74.8 ± 6.1 μmol/L) ($P < 0.05$) and at day 28 (treated 48.5 ± 1.6μmol/L and control 96.2 ± 16.7 μmol/L) ($P < 0.001$) was significantly less in treated animals (Fig. 2B).

Glomerular and tubulointerstitial scarring were scored and ranked respectively in randomized hematoxylin and eosin–stained sections. At day 28, the mean glomerular quadrant scar score was significantly less in the treated animals compared to the controls (treated 0.5 and control 2.2) ($P < 0.01$). The treated group also showed significantly less tubulointerstitial scarring than the controls (treated 0.6 and control 2.4) ($P < 0.01$) (Fig. 3).

Quantitative assessment of fibrosis was performed using an immunohistochemical stain for type IV collagen. There was a significant decrease in the percentage cortical area stained at day 28 in treated animals (treated 1.9 ± 1.1% and control 9.3 ± 2.2%) ($P < 0.05$) (Fig. 4). α-SMA is an actin isoform expressed in smooth muscle cells, fibroblasts, and mesangial cells undergoing myofibroblastic transformation. This phenotype is associated with matrix synthesis, and therefore acts as a marker of future fibrosis. There was a significant decrease in glomerular α-SMA at day 28 in treated animals (treated 2.3 ± 0.6% and control 11.5 ± 2.3%) ($P < 0.01$). Another marker of renal fibrosis is the ED(A) splice variant domain of fibronectin (FN). Quantitation of glomerular staining for ED(A)FN showed that there was reduced expression in

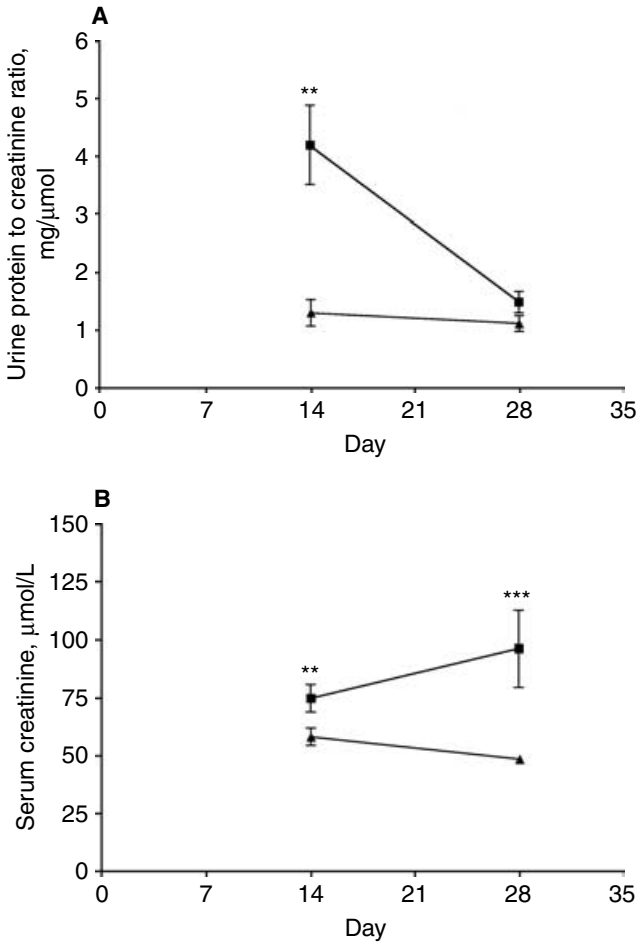

Fig. 2. Experiment 3. Renal function in rats treated with anti-tumor necrosis factor-α (TNF-α) monoclonal antibody from day 4 until day 28. (*A*) Urinary protein to creatinine ratio was lower in animals treated from day 4 until day 28, at day 14 (**$P < 0.01$) compared with controls. (*B*) Serum creatinine was lower in animals treated from day 4 until day 28, at day 14 (**$P < 0.01$) and day 28 (***$P < 0.001$) compared with controls. Anti-TNF-α monoclonal antibody (▲) and control (■).

the treated animals compared to controls (treated 12.3 ± 2.5% and control 19.6 ± 3.2%) ($P = 0.09$). Anti-TNF-α monoclonal antibody treatment did not have an effect on glomerular infiltration by macrophages (treated 3.6 ± 0.7 and control 5.0 ± 0.7 macrophages/glomerulus) or CD8-positive cells. However, tubulointerstitial counts showed significantly lower numbers of interstitial macrophages in the treatment group (treated 5.3 ± 1.2 and control 20.5 ± 1.9 macrophages/interstitial area) ($P < 0.01$).

Urinary and serum MCP-1 levels were measured using a direct ELISA. Animals treated with anti-TNF-α monoclonal antibody had significantly reduced amounts of MCP-1 in their urine in comparison to control animals at day 14 (treated 1.6 ± 0.23 and control 4.4 ± 1.0 ng MCP-1/mmol creatinine) ($P < 0.05$) and 28 (treated 0.7 ± 0.14 and control 3.5 ± 0.89 ng MCP-1/mmol creatinine) ($P < 0.01$). There was no significant difference in serum MCP-1 levels at day 28.

Rabbit and rat IgG deposition in the glomeruli were quantitated by direct immunofluorescence, and were similar in both groups. In addition, circulating levels of rat antirabbit Ig in the serum were quantitated by ELISA, and were similar in both groups (treated 2.4 ± 0.12 and control 1.8 ± 0.22 OD units). This indicates that blockade of TNF-α did not affect glomerular deposition of administered rabbit IgG, or the animals' immune response (rat IgG) to the planted rabbit immunoglobulin.

Effect of delayed treatment with anti-TNF-α monoclonal antibody in glomerulonephritis

In experiment 4, rats were treated from day 14 to day 28 with anti-TNF-α monoclonal antibody. Treatment was started at the time of maximal crescent formation in order to mimic the clinical situation in which patients often present with severe disease.

There was no difference between the groups in urine protein to creatinine ratio at day 28 (treated 2.09 ± 0.18 and control 1.64 ± 0.11 mg/μmol). After 2 weeks of treatment, at day 28, treated animals had a lower level of serum creatinine than controls (treated 85.0 ± 3.2 μmol/L and control 134.0 ± 12.5 μmol/L) ($P < 0.05$) (Fig. 5). This was associated with reduced tubulointerstitial scarring in rats killed at day 28. Semiquantitative assessment by ranking showed a significant reduction in interstitial scarring in treated rats ($P < 0.05$). Quantitation of glomerular inflammatory cells showed no significant difference in numbers of glomerular macrophages (treated 3.2 ± 1.0 and control 3.3 ± 0.4 macrophages/glomerulus) or CD8-positive cells between treated and control groups. Rabbit and rat IgG deposition in the glomeruli were similar in both groups. Levels of circulating rat antirabbit Ig were also similar in both groups (treated 2.0 ± 0.16 and control 1.7 ± 0.11 OD units).

DISCUSSION

Blockade of TNF-α is increasingly used in predominantly T-cell–mediated autoimmune diseases such as Crohn's disease and rheumatoid arthritis [7, 8]. Treatment with anti-TNF antibodies in these diseases has proven to be effective in the majority of patients. The success of TNF inhibition has encouraged research in other chronic inflammatory diseases. We hypothesized that blocking TNF-α with a monoclonal antibody would reduce both inflammation and fibrosis in crescentic glomerulonephritis.

Anti-TNF-α therapy was initially investigated in the acute phase of the nephrotoxic nephritis model, as this is when inflammation is predominant. Anti-TNF-α antibody was effective in reducing fibrinoid necrosis and

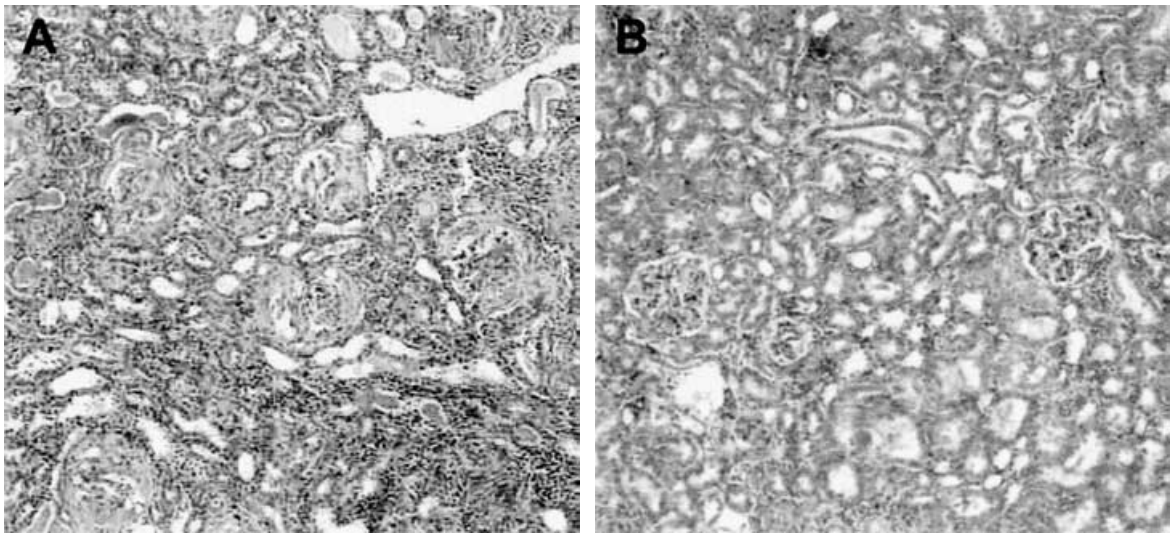

Fig. 3. Experiment 3. Histology of kidney sections from rats treated with anti-tumor necrosis factor-α (TNF-α) monoclonal antibody from day 4 until day 28. (*A*) Histology from a control animal showing marked glomerular scarring with fibrous crescents, tubulointerstitial scarring with tubular atrophy, and tubulointerstitial inflammation (hematoxylin and eosin ×10). (*B*) Histology from a treated animal showing reduced glomerular and tubulointerstitial scarring, less tubular atrophy, and reduced interstitial inflammation compared with control (hematoxylin and eosin ×10).

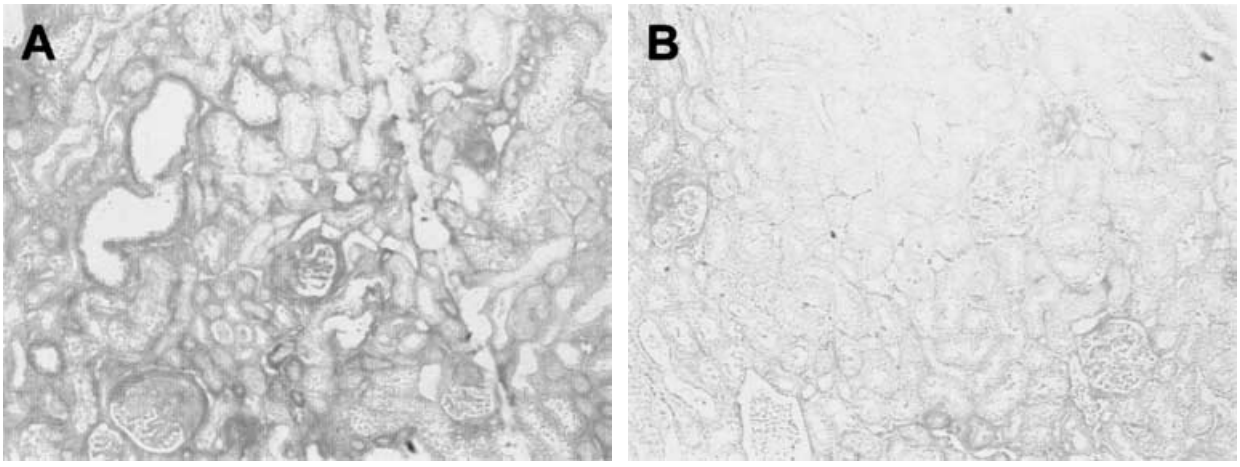

Fig. 4. Experiment 3. Immunohistochemistry for type IV collagen in kidneys from rats treated with anti-tumor necrosis factor-α (TNF-α) monoclonal antibody from day 4 until day 28. (*A*) Cortical type IV collagen immunohistochemistry from a control animal showing marked deposition (×20). (*B*) Cortical type IV collagen immunohistochemistry from an anti-TNF-α monoclonal antibody–treated animal showing reduced deposition compared with control (×20).

glomerular macrophage infiltration when given from day 0 to day 7. Effectiveness was also demonstrated when antibody was given from day 0 to day 14, or from day 4 to day 14, with a reduction in glomerular crescents and preservation of renal function. Of note, the second of these studies showed that treatment was effective even when started at day 4, when there is maximum glomerular macrophage infiltration [22].

These experiments were extended to investigate whether suppression of the initial glomerular inflammation would lead to a sustained improvement in renal function and reduction in scarring. Treatment was begun at day 4 and continued until day 28, at which time kidneys in untreated animals show considerable glomerular and tubulointerstitial scarring. Beneficial effects were sustained in later stages of the disease, as shown by the finding that renal function was preserved, and markers of fibrosis, including type IV collagen and α-SMA, were significantly reduced at day 28. Urinary levels of the chemokine MCP-1 were also significantly reduced. When treatment was delayed to day 14 of disease, at which time there is severe glomerulonephritis with maximal crescent formation, blockade of TNF-α was still effective. There was preservation of renal function and reduction in interstitial scarring, but no effect on glomerular scarring.

Previous studies in rodent models of crescentic glomerulonephritis have involved the blockade of TNF-α with soluble TNFR p55 or TNF binding protein, which

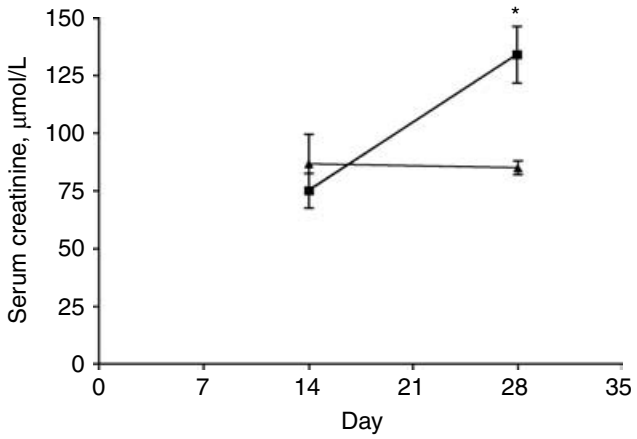

Fig. 5. Experiment 4. Renal function in rats treated with anti-tumor necrosis factor-α (TNF-α) monoclonal antibody from day 14 until day 28. Serum creatinine was lower in treated animals at day 28 ($^*P < 0.05$) compared with controls. Anti-TNF-α monoclonal antibody (▲) and control (■).

bind circulating TNF [14–16]. It is possible that the effects of soluble receptors may not be the same as monoclonal antibodies. Antibody treatment will bind both soluble and membrane-bound TNF, while the soluble receptor binds soluble TNF alone. In addition, binding of membrane bound TNF by antibody therapy may promote antibody-dependent cell cytotoxicity and apoptosis of activated effector T cells and macrophages, which may be an important mechanism of action. In support of this difference, in Crohn's disease the chimeric anti-TNF antibody, infliximab, shows good efficacy in treatment; however, the soluble TNF receptor, etanercept, has not proven to be so effective [27, 28]. Previous experimental studies of TNF blockade in glomerulonephritis have another limitation, as they were short term [14, 15] and thus less directly relevant to progression of disease in patients. Our present work addresses the role of TNF-α in long-term studies, by investigating the treatment and prevention of nephrotoxic nephritis at more clinically relevant stages.

TNF-α is paramount in the inflammatory response, as it stimulates a cascade of events, including the production of other proinflammatory cytokines (e.g., IL-1, IL-6, and MIF), chemokines (e.g., MCP-1), and growth factors (e.g., TGF-β). It has long been considered that the source of TNF-α in renal inflammation was infiltrating macrophages; however, recent studies using TNF-α −/− chimeric mice have shown that production from intrinsic renal cells is also important [18]. TNF-α synthesized by mesangial cells and podocytes acts to enhance inflammatory cell recruitment, stimulate mesangial cell production of TGF-β, and promote glomerular fibrin deposition by inducing release of tissue factor.

There is mounting evidence that TNF-α is also contributory to the development of fibrosis. TNF-α is widely accepted to play a major role in mediating interstitial pulmonary fibrosis. In a model of bleomycin-induced lung disease, anti-TNF-α treatment has been shown to be effective in reducing pulmonary fibrosis, and in lowering MCP-1 and TGF-β mRNA levels in the lung [29]. In another study, using mice deficient in both TNFR1 and TNFR2, exposure to asbestos fibers increased levels of TNF-α gene and protein expression; however, these mice were protected from developing fibroproliferative lesions by a lack of receptor signaling [30]. These studies demonstrate a fibrogenic role for TNF-α, by inducing cytokines and profibrotic factors that amplify the inflammatory response and progression to fibrosis.

During disease progression in glomerulonephritis, cellular infiltration into the kidney allows interaction of inflammatory cells with glomerular cells and fibroblasts, thus stimulating the synthesis of extracellular matrix and leading to glomerular and tubulointerstitial scarring. The development of proteinuria activates proximal tubular epithelial cell NF-κB and induces secretion of MCP-1 into the interstitium from an early time point in experimental and human glomerulonephritis [31, 32]. MCP-1 can also be produced by renal fibroblasts, as demonstrated by in vitro coculture experiments [33]. An overall increase in MCP-1 subsequently results in the recruitment of circulating mononuclear cells to within the tubulointerstitial area. In the present study, treatment with anti-TNF-α antibody from day 4 to day 28, reduced proteinuria, together with a reduction in tubulointerstitial macrophages and urinary MCP-1 levels. This reduction in urinary MCP-1, in the presence of similar serum levels, may reflect reduced renal production of MCP-1, which could be a potential mechanism in preventing tubulointerstitial fibrosis in glomerulonephritis.

Our results support the proposition that TNF-α is centrally involved in the pathogenesis of glomerulonephritis, and is thus a key therapeutic target. Neutralization of endogenous TNF-α with anti-TNF-α monoclonal antibody in this model of nephrotoxic nephritis reduces glomerular inflammation, crescent formation, and interstitial scarring, and prevents renal failure. These studies suggest that TNF-α is important in both the early inflammatory phase and the later scarring phase of the disease. It seems likely that the effects of TNF blockade in glomerulonephritis are at least partly due to the reduced activity of downstream mediators, such as MCP-1. It remains unclear whether TNF-α blockade reduces renal fibrosis predominantly by preventing inflammation, or whether it is affecting both phases of the disease by separate mechanisms. Our delayed studies suggest, but do not prove, a direct effect on renal scarring, as previously observed in TNFR knockout mice. Regardless of the exact mechanism, our work clearly demonstrates that blockade of TNF-α is effective in experimental crescentic glomerulonephritis. Preliminary studies suggest a benefit of this approach in patients with glomerulonephritis due to

systemic vasculitis [34], and randomized clinical trials are clearly needed.

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