The Pathogenic Role of Macrophage Migration Inhibitory Factor in Immunologically Induced Kidney Disease in the Rat

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Summary

Macrophage migration inhibitory factor (MIF) plays a pivotal role in the inflammatory response in endotoxemia and in the delayed-type hypersensitivity response, but its potential as a regulator of immunologically induced disease is unknown. We have addressed this issue by administering a neutralizing anti-MIF antibody in a rat model of immunologically induced crescentic anti-glomerular basement membrane (GBM) glomerulonephritis. Six individual experiments using paired inbred littermates were performed. Rats were primed with rabbit immunoglobulin on day −5 and then injection with rabbit anti–rat GBM serum on day 0. Pairs of animals were treated with anti-MIF or a control monoclonal antibody from the time of anti-GBM serum administration until being killed 14 d later. Control antibody-treated animals developed severe proteinuria and renal function impairment with severe histological damage due to marked leukocytic infiltration and activation within the kidney. In contrast, anti-MIF treatment substantially reduced proteinuria, prevented the loss of renal function, significantly reduced histological damage including glomerular crescent formation, and substantially inhibited renal leukocytic infiltration and activation (all $P < 0.001$ compared with control treatment). Inhibition of renal disease by anti-MIF treatment was attributed to preventing the marked upregulation of interleukin-1β, leukocyte adhesion molecules including intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, and inducible nitric oxide synthase expression seen in the control antibody-treated animals. This inhibition of progressive renal injury was mirrored by the complete suppression of the skin delayed-type hypersensitivity response to the challenge antigen (rabbit IgG). Interestingly, anti-MIF treatment did not effect the secondary antibody response or immune deposition within the kidney, indicating that MIF participates in cellular-based immunity in this primed macrophage-dependent anti-GBM glomerulonephritis. In conclusion, this study has demonstrated a key regulatory role for MIF in the pathogenesis of immunologically induced kidney disease. These results argue that blocking MIF activity may be of benefit in the treatment of human rapidly progressive glomerulonephritis, and suggest that MIF may be important in immune-mediated disease generally.

Macrophage migration inhibitory factor (MIF)[1] was originally described as a product of activated T cells that inhibited the random migration of guinea pig peritoneal macrophages in vitro and promoted macrophage accumulation in the delayed-type hypersensitivity (DTH) reaction (1, 2). Recent studies using neutralizing antibodies have established the central role of MIF in the DTH response, as an important mediator of endotoxic shock, and as a counter regulator of glucocorticoid action (3–5). MIF has also been shown to play an important role in primary antigenic and mitogenic stimulation of T cell activation and T cell–dependent antibody production (6). These findings demonstrate that MIF is a crucial mediator of the inflammatory and immune response and, therefore, is likely to be a key regulator of immune-mediated disease, although this remains to be proven.

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[1] *Abbreviations used in this paper:* APAAP, alkaline phosphatase anti-alkaline phosphatase; DIG, digoxigenin; DTH, delayed-type hypersensitivity; GBM, glomerular basement membrane: GCS, glomerular cross-section; H&E, hematoxylin and eosin; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; MIF, macrophage migration inhibitory factor; PAP, peroxidase anti-peroxidase; PAS, periodic acid–Schiff reagent; PLP, paraformaldehyde–lysine–periodate

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To investigate whether MIF is a key mediator of immune-mediated disease, we used a neutralizing antibody to block the action of MIF in a rat model of accelerated anti-glomerular basement membrane (GBM) glomerulonephritis. This model was chosen because we have previously described a marked upregulation of renal MIF expression, which correlated with macrophage accumulation and progressive renal injury (7). Therefore, the aims of the present study were to determine the following: (*a*) whether MIF plays a pathological role in immune-mediated renal injury, and (*b*) the mechanisms by which MIF may participate in the disease process.

Materials and Methods

Antibodies and Probes. The following mouse mAbs were used: IIID9, mouse anti–murine MIF mAb raised to purified recombinant mouse MIF (Metz, C.N., and R. Bucala et al., manuscript in preparation); OX-1; anti-rat CD45, leukocyte common antigen (8); ED1, anti-rat CD68 labels most monocytes and macrophages (9); R73, recognizes a nonpolymorphic epitope of the αβ TCR (10); NDS 61, anti-rat CD25, p55 chain of the rat IL-2R (11); 1A29, anti-rat intercellular adhesion molecule-1 (ICAM-1; CD54) (12); 5F10, anti-rat vascular cell adhesion molecule-1 (VCAM-1, CD106) (supplied by Biogen, Inc., Cambridge, MA); MCA 1397, anti-rat recombinant IL-1β (Serotec, Oxford, UK) (13); N32030, anti-rat inducible nitric oxide synthase (iNOS) (Transduction Laboratories, Lexington, KY). In addition, a mouse anti–human CD45R mAb (73.5) that does not react with rat tissues was used as a negative control. Peroxidase and alkaline phosphatase–conjugated goat anti–mouse IgG, mouse peroxidase anti-peroxidase complexes (PAP), and mouse alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) were all purchased from Dakopatts (Glostrup, Denmark). FITC-conjugated goat anti–rat IgG, C3, and fibrinogen or FITC-conjugated sheep anti–rabbit IgG were purchased from Nordic (the Netherlands).

A 420-bp fragment of mouse MIF cDNA cloned into pBluescript (Strategene, La Jolla, CA) (14) was used to prepare digoxigenin (DIG)-labeled cRNA probes for in situ hybridization according to the protocol of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). In addition, the MIF cDNA and a β-actin cDNA were random primed with [^{32}P]dCTP for Northern blot analysis.

Experimental Disease Model. Passive accelerated anti-GBM disease was induced in inbred male Sprague-Dawley rats (150–180 g) (Monash Animal Services, Melbourne, Australia) as previously described (15). Each experiment consisted of a matched pair of inbred littermates immunized at the same time by subcutaneous injection of 5 mg normal rabbit IgG in Freund's complete adjuvant followed 5 d later (day 0) by intravenous injection of 10 ml/kg bodyweight rabbit anti–rat GBM serum (12.5 mg IgG/ml). Starting 2 h before the administration of anti-GBM serum, one rat from each pair was treated by an intraperitoneal injection of 5 mg/kg of either anti-MIF mAb (IgG1) or a control irrelevant mAb antibody (73.5; IgG1). Antibody administration was repeated every second day until being killed on day 14. A total of six littermate pairs were examined. Blood and 24-h urine collections were taken on days 0, 1, 7, and 14. Urinary protein excretion was determined using the Manual Ponceau Red method. Concentrations of serum and urine creatinine, and serum urea were determined using the standard Jaffe rate reaction (alkaline picrate) or NED/OPA assay, respectively. In addition, heparinized blood samples were taken at time of death and white blood cells were analyzed by Coulter Counter (Cell Dyne). All analyses were performed by the Departments of Biochemistry and Haematology, Monash Medical Centre.

Skin Delayed-type Hypersensitivity Response. 24 h before being killed, each animal was given three separate intradermal injections of 0.1 ml each of rabbit IgG (1 mg/ml), horse IgG (1 mg/ml), and PBS. Skin thickness at the time of killing was measured using engineer's callipers.

Histopathology. Tissues were fixed in 4% buffered formalin and 4 μm paraffin sections were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) reagent. Glomerular hypercellularity was graded on the basis of the total glomerular cell count/glomerular cross-section (gcs) scored in 100 glomerular cross-sections per animal in H&E-stained sections and ranked as previously described (16): 0, normal (less than 50 cells/gcs); 1, mild (60–80 cells/gcs); 2, moderate (80–120 cells/gcs); 3, severe hypercellularity (more than 120 cells/gcs). The percentage of glomeruli exhibiting segmental and/or global sclerosis or crescent formation was also scored in 100 glomeruli per animal in PAS-stained sections. A point-counting technique was used to quantitate tubulointerstitial damage (tubular atrophy, leukocytic infiltration, and fibrosis) in at least 50 high-power cortical fields in PAS stained sections. Data is presented as the mean of six animals ± SEM. Analysis was performed on blinded slides.

Measurement of the Humoral Immune Response. Circulating levels of rat IgG reactive with rabbit immunoglobulin was quantitated by a sandwich ELISA, as previously described (16–17). Deposition of immune reactants within the kidney was assessed by direct immunofluorescence. Tissues were snap frozen in liquid nitrogen and 4-μm cryostat sections were stained with FITC-conjugated polyclonal antibodies to rat IgG, C3, fibrinogen, or rabbit IgG. Semiquantitation of rabbit IgG, rat IgG, C3, and fibrinogen deposition was determined in tissue sections using an antibody titration method (16–17). Sections from each tissue were incubated with serial two-fold dilutions of each antibody. Slide were blinded and the antibody dilution at which stained became undetectable scored. Results are expressed in terms of antibody dilution.

Immunohistochemistry. One-color immunohistochemical staining using mAbs to CD45, TCR, CD68, IL-2R, ICAM-1, and VCAM-1 was performed on cryostat sections of tissues fixed in 2% paraformaldehyde–lysine–periodate (PLP), as previously described (15–17). In brief, sections were preincubated with 10% FCS and 10% normal goat serum in PBS for 20 min, drained, labeled with mouse mAb for 60 min, washed (3×) in PBS, and then endogenous peroxidase inactivated by incubation in 0.3% H_2O_2 in methanol. Sections then were washed in PBS, incubated with peroxidase-conjugated goat anti–mouse IgG, washed in PBS, incubated with mouse PAP complexes, and developed with 3,3-diaminobenzidine to produce a brown color.

Double immunohistochemical staining was performed on either PLP-fixed cryostat sections or formalin-fixed paraffin sections using a previously described microwave-based method (18). In brief, sections were treated by 2× 5 min of microwave oven heating in 0.01 M sodium citrate (pH 6.0) at 2450 MHz and 800 W. This treatment markedly enhanced antibody access to MIF, CD68, IL-1β, and iNOS. Sections were then precincubated and stained with ED1 or anti-iNOS mAbs using the three-layer PAP method as described above. After a second round of microwave oven heating to denature the bound immunoglobulins, thereby preventing antibody cross-reactivity (19), sections were preincubated and then labeled with mAbs to MIF, IL-1β, or CD68 (ED1) overnight at 4°C. After washing in PBS, sections were incubated sequentially with alkaline phosphatase–conjugated goat anti–mouse IgG and mouse APAAP, and then developed with Fast blue BB Salt (Ajax Chemicals, Melbourne, Australia) or nitroblue tetrazolium (NBT)/X-phosphate. Sections were counterstained with PAS and mounted in an aqueous medium.

In Situ Hybridization. In situ hybridization was performed on 4-μm paraffin sections of formalin-fixed tissue using a newly developed microwave-based protocol as described in detail elsewhere (7, 19). After dewaxing, sections were treated with a microwave oven for 2× 5 min as outlined above, incubated with 0.2 M HCl for 15 min, followed by 1% Triton X-100 for 15 min, and then digested for 20 min with 10 μg/ml proteinase-K at 37°C (Boehringer Mannheim GmbH, Mannheim, Germany). Sections then were washed in 2× SSC, prehybridized, and then hybridized with 0.3 ng/μl digoxigenin-labeled sense or anti-sense cRNA probe overnight at 42°C in a hybridization buffer containing 50% deionised formamide, 4× SSC, solution, 1 mg/ml salmon sperm DNA (Sigma), and 1 mg/ml yeast tRNA. Sections were washed finally in 0.1× SSC at 42°C and the hybridized probe detected using alkaline phosphatase–conjugated sheep anti-digoxigenin F(ab) fragments and color development with NBT/X-phosphate. In addition, some sections were treated with microwave oven heating again and then double-stained with the ED1 mAb using the three-layer PAP method described above. Sections were counterstained with PAS and mounted in an aqueous medium.

Northern Blotting. Total cellular RNA was extracted from one-half of one kidney using RNAzol-B according to the instructions of the manufacturer (Tel-Test Inc., Friendswood, TX). RNA samples (20 μg) were denatured with glyoxal and dimethylsulphoxide, size fractionated on 1.2% agarose gels, and capillary-blotted onto Hybond-N membranes (Amersham International, Buckinghamshire, UK). Membranes were hybridized overnight at 42°C with ^{32}P-labeled MIF cDNA in 5× SSPE, 50% formamide, 0.2 mg/ml herring sperm DNA, 0.2 mg/ml yeast RNA, 0.5% SDS, and 5× Denhardt's solution. After hybridization, membranes were washed finally in 0.2× SSC, 0.1% SDS at 42°C. Autoradiography was performed at room temperature for 4 h using DuPont Reflection films and intensifying screens. Blots were stripped and reprobed for β-actin.

Quantitation of Tissue Staining. A standard semiquantitative method was used to quantitate both glomerular and tubulointerstitial infiltrate as previously described (7, 15–17). Cells labeled by each mAb or the MIF cRNA probe were counted in high power fields (×40) of 20 consecutive glomerular cross-sections (gcs) for each animal and expressed as the number of labeled cells per gcs. Glomerular staining with mAb to ICAM-1, VCAM-1, and IL-1β was graded on a scale of 0, no staining; 1, <25% stained; 2, 25–50% stained; 3, >50% of the gcs stained. To assess tubulointerstitial staining, cortical areas were selected at random. The number of labeled interstitial cells or tubular epithelial cells was counted in 20 consecutive high-power fields by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope, and expressed as cells/mm^2. These fields progressed from the outer to inner cortex, avoiding only large vessels, glomerular, and immediate periglomerular areas. For each tissue, the same area was examined in serial sections labeled with different mAbs. No adjustment of the interstitial cell count was made for tubules or the luminal space. Data is expressed as the mean for six animals ± SEM. All counting was performed on blinded slides.

Measurement of Glomerular MIF and Nitric Oxide Production. Glomeruli were isolated by differential sieving technique in which a half kidney from each animal was placed in RPMI-1640 medium with 5% FCS, diced finely, gently pressed through a 250-μm wire mesh, and poured through a 106-μm and then a 75-μm wire mesh. Glomeruli remaining on the top of the 75-μm mesh were washed extensively with RPMI, 5% FCS and collected. Isolated glomeruli were >95% pure as assessed by phase-contrast microscopy. Glomeruli were cultured at 3,000 glomeruli/ml in RPMI 1640, 5% FCS in 5% CO_2 at 37°C for 24 h with or without 2 μg/ml LPS and the supernatant harvested. The concentration of MIF in supernatant samples was quantitated by a sandwich ELISA (5), and the concentration of nitrite was measured using a standard Griess assay (20).

Statistical Analysis. Parametric or nonparametric data obtained from this study was analyzed by an unpaired two-sided Student's *t*-test or a Mann-Whitney U test using the Complete Statistical Systems program (Statsoft, Tulsa, OK).

Results

Renal Function and Histological Damage. The induction of accelerated anti-GBM disease in rats treated with an irrelevant control mAb resulted in the development of severe proteinuria and a significant loss of renal function as assessed by increased levels of serum creatinine and urea and a reduction in the glomerular filtration rate indicated by creatinine clearance (Fig. 1). Although anti-MIF mAb treatment did not affect the induction of mild proteinuria on day 1, it did prevent the development of severe proteinuria over days 1–14 (Fig. 1 *a*). Importantly, normal renal function was maintained throughout the experimental period in anti-MIF mAb treated animals. In addition, anti-MIF treatment had no effect upon circulating white blood cell numbers (data not shown).

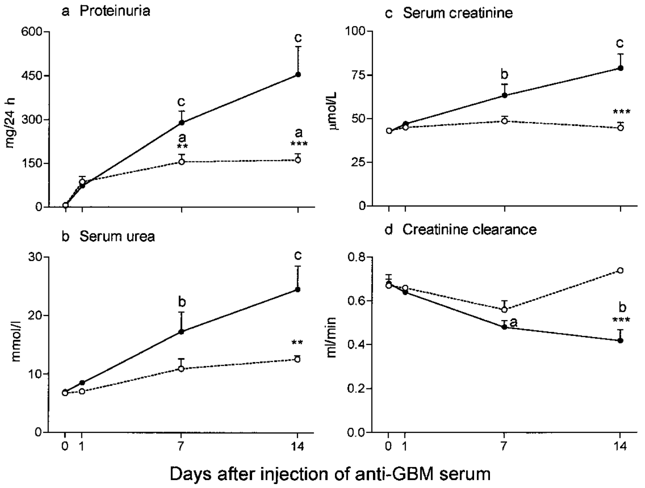


Figure 1. Effect of anti-MIF treatment on renal function in rat anti-GBM disease. The effect of anti-MIF (*open circles*; *dotted line*) and irrelevant control (*closed circles*; *solid line*) antibody treatment on the following: (*a*) urinary protein excretion, (*b*) serum urea levels, (*c*) serum creatinine levels, and (*d*) creatinine clearance, was assessed at different times after injection of anti-GBM serum. Data is as the mean ± SEM for six animals. **P <0.01, ***P <0.001 versus time-matched control antibody-treated animals, and; $^a P$ <0.05, $^b P$ <0.01, $^c P$ <0.001 versus normal rats (day 0) by an unpaired two-sided Student's *t*-test.

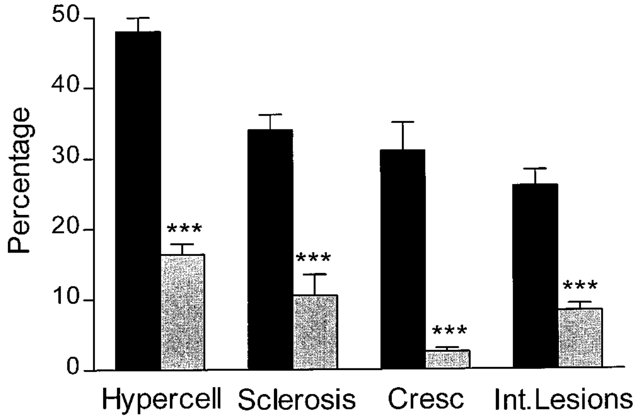


Figure 2. Effect of anti-MIF antibody treatment on histological damage in rat anti-GBM disease. The effect of anti-MIF (*gray bars*) and irrelevant control (*closed bars*) antibody treatment on glomerular hypercellularity (*Hypercell*), glomerular sclerosis (*Sclerosis*), glomerular crescents formation (*Cresc*), and tubulointerstitial lesions (*Int.Lesions*) was determined on day 14 of rat anti-GBM disease. Data is as the mean ± SEM for six animals. ***$P < 0.001$ versus control antibody treated by an unpaired two-sided Student's *t*-test.

In accord with the decline in renal function, control antibody-treated animals displayed marked histological damage on day 14 of anti-GBM glomerulonephritis, exhibiting moderate to severe glomerular hypercellularity, severe focal/segmental lesion and crescent formation, and marked tubulointerstitial lesions (Fig. 2). All of these indices of histological damage were substantially inhibited, particularly glomerular crescent formation, by anti-MIF mAb treatment (Fig. 2).

Adhesion Molecule Expression and Leukocyte Infiltration and Activation. There was a prominent glomerular and interstitial leukocytic infiltrate on day 14 in control antibody-treated animals (Fig. 3, *a* and *b*). The glomerular infiltrate was composed mainly of $ED1^+$ macrophages, whereas there was a mixed $ED1^+$ macrophage and $R73^+$ T cell infiltrate in the cortical interstitium. Significant activation of the T cell infiltrate was indicated by the presence of many IL-2R–positive cells. Associated with leukocyte infiltration was a substantial increase in glomerular and interstitial expression of the adhesion molecules ICAM-1 and VCAM-1 (Fig. 3, *c* and *d*). In contrast, treatment of rat anti-GBM glomerulonephritis with anti-MIF mAb reduced glomerular macrophage infiltration by ~60% (Fig. 3 *a*), and largely abrogated interstitial macrophage and T cell infiltration and activation (Fig. 3 *b*). The marked inhibition of leukocytic infiltration was accompanied by a reduction in glomerular and interstitial expression of ICAM-1 and VCAM-1 (Fig. 3).

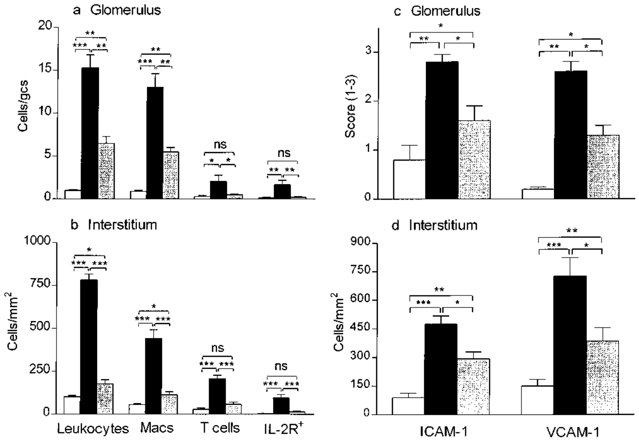
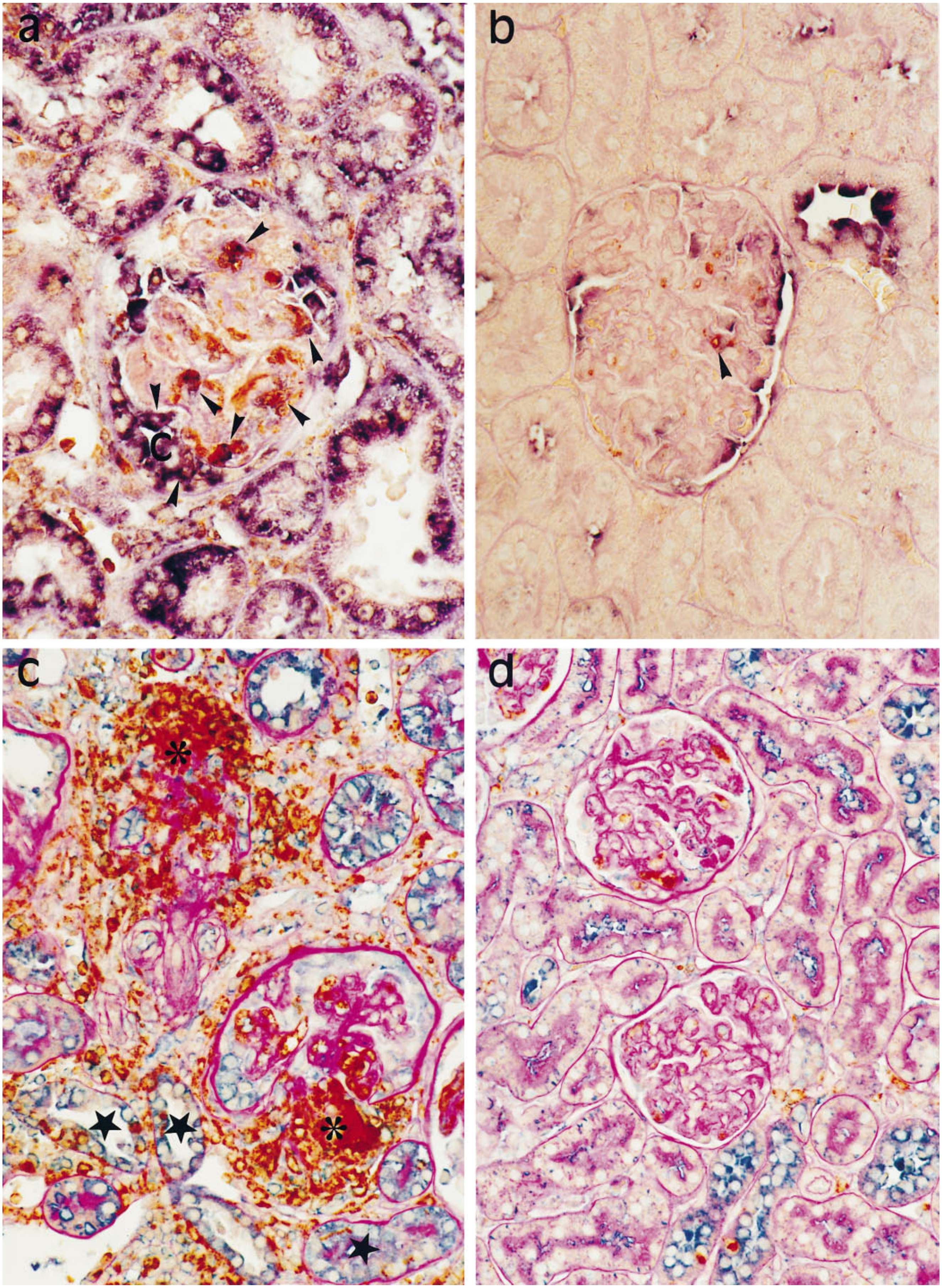


Figure 3. Effect of anti-MIF antibody treatment on leukocyte infiltration and adhesion molecule expression in rat anti-GBM disease. The effect of anti-MIF (*gray bars*) and irrelevant control (*closed bars*) antibody treatment on glomerular and tubulointerstitial leukocytic infiltration, activation, and ICAM-1 or VCAM-1 expression was analyzed on day 14 of rat anti-GBM disease using an unpaired two-sided Student's *t*-test or a Mann-Whitney U test for glomerular ICAM-1 and VCAM-1 expression. Data is as the mean ± SEM for six animals. Normal rats (*open bars*). ns, no significant difference, *$P < 0.05$, **$P < 0.001$, ***$P < 0.001$.

MIF mRNA and Protein Expression. There was a dramatic increase in renal MIF expression in control antibody-treated anti-GBM glomerulonephritis as demonstrated by Northern blotting, in situ hybridization, and immunohistochemistry staining (Figs. 4 and 5). Indeed, Northern blot analysis showed that there was a significant increase in total kidney MIF mRNA in control-treated animals compared with the animals treated with the anti-MIF mAb (MIF mRNA/β-actin: 1.9 ± 0.15 versus 0.85 ± 0.1, $P < 0.01$). In particular, both combined in situ immunohistochemistry and double immunostaining showed upregulation of glomerular and tubular MIF expression in areas of focal macrophage accumulation (Fig. 4, *a* and *c*). This upregulation of MIF mRNA and protein expression by both intrinsic kidney cells and infiltrating macrophages was largely prevented by anti-MIF mAb treatment (Fig. 4, *b* and *d*; Fig. 5). This was also evident in measuring MIF secretion by isolated glomeruli. Control treated animals showed a significant increase in MIF secretion compared with both normal (2434 ± 98 versus 1955 ± 169 pg/ml; $P < 0.05$). and anti-MIF–treated animals (2434 ± 98 versus 1180 ± 100 pg/ml; $P < 0.001$). Interestingly, anti-MIF treatment also reduced glomerular MIF level to below that constitutively secreted by glomeruli isolated from normal rats (1180 ± 100 versus 1955 ± 169 pg/ml; $P < 0.01$).

Figure 4. Effect of anti-MIF treatment on renal MIF mRNA and protein expression in rat anti-GBM disease. Combined in situ hybridization and immunohistochemistry (*a* and *b*) and double immunostaining (*c* and *d*) illustrate the marked upregulation of glomerular and tubulointerstitial MIF mRNA (*dark blue*) and protein (*blue*) expression in association with local macrophage (*brown*) accumulation, severe tubulitis (*stars*), and tissue injury (asterisk) in control antibody-treated anti-GBM disease (*a* and *c*), In contrast, anti-MIF antibody treatment substantially reduced renal MIF mRNA and protein, as illustrated in *b* and *d*, together with inhibition of macrophage accumulation. In addition, strong MIF expression by macrophages (*purple*; *arrow heads*) seen in control antibody-treated animals (*a* and *c*) is almost completely abolished by anti-MIF treatment (*b* and *d*). The photomicrographs from the anti-MIF–treated animals represent the areas of the kidney with least inflammatory involvement. Sections were counterstained with PAS. Magnification, ×320.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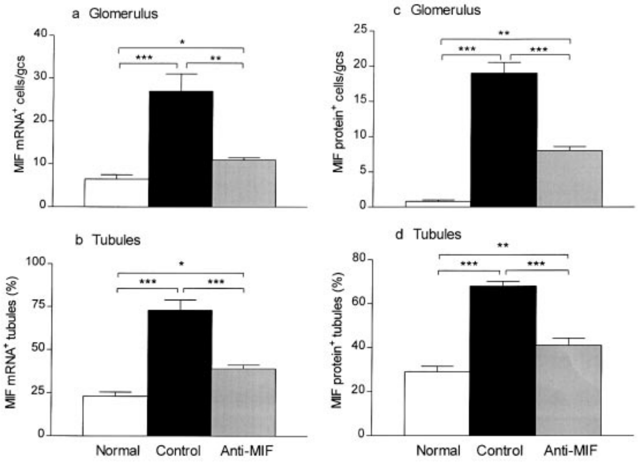


Figure 5. Quantitation of MIF expression in rat anti-GBM disease. The number of MIF mRNA-positive cells (*a* and *b*) and MIF protein stained cells (*c* and *d*) were scored in normal rat kidney (*open bars*), and in control (*closed bars*) and anti-MIF (*gray bars*) antibody treated anti-GBM disease. Data is expressed as the mean ± SEM for six animals. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

IL-1β Expression. Antibody staining of tissue sections showed a marked increase in glomerular IL-1β expression in control antibody-treated anti-GBM glomerulonephritis. There was also a striking increase in the intensity of anti–IL-1β mAb staining of cortical tubular epithelial cells and an increase in the percentage IL-1β–positive tubules (Fig. 6 *a*; Fig. 7). Double immunostaining demonstrated that many infiltrating macrophages, including multinucleated giant cells within both glomeruli and tubulointerstitium, were also IL-1β positive (see Fig. 6 *a*). In contrast, this increase in IL-1β expression by both intrinsic kidney cells and macrophages was largely abrogated by anti-MIF antibody treatment (see Fig. 6 *b*; Fig. 7).

iNOS Expression. The induction of anti-GBM glomerulonephritis in control mAb-treated animals caused a dramatic increase iNOS expression by intrinsic glomerular and interstitial cells and tubular epithelial cells (see Fig. 6 *c*; Fig. 8). Double immunostaining showed that many ED1$^+$ macrophages expressed iNOS (see Fig. 6 *c*). Treatment with anti-MIF mAb resulted in a marked inhibition of glomerular, interstitial, and tubular iNOS expression; in particular, macrophage iNOS expression (see Fig. 6 *d*; Fig. 8).

Glomerular nitric oxide production, as measured by nitrite levels in LPS-stimulated cultured glomeruli, was increased in control mAb treated animals compared with normal rats (35.5 ± 4.6 versus 8.4 ± 0.8 μmol/l; $P < 0.001$). This was significantly reduced by anti-MIF treatment (21.7 ± 1.9 versus 35.5 ± 4.6 μmol/l; $P < 0.05$).

Humoral Immune Response. Anti-MIF treatment had no effect on serum levels of rat anti–rabbit IgG antibody in anti-GBM glomerulonephritis (Fig. 9). In addition, semiquantitative assessment of immunofluorescence staining found that there was no difference between control and anti-MIF–treated animals in the deposition of rabbit IgG (14000 ± 1309 versus 12000 ± 1789; $P > 0.3$), rat IgG (1125 ± 206 versus 1000 ± 225; $P > 0.6$) and rat C3 (7429 ± 571 versus 6667 ± 1978; $P > 0.6$) within the kidney.

Skin DTH Response. Animals treated with the control mAb mounted a vigorous skin DTH response to rabbit IgG, but not to the irrelevant horse IgG. There was marked skin swelling and a pronounced cellular immune response involving macrophage infiltration, T cell recruitment, and activation and induction of iNOS expression at the site of rabbit IgG injection (Fig. 10). Treatment of anti-GBM glomerulonephritis with anti-MIF mAb abrogated skin swelling in response to rabbit IgG injection and significantly suppressed the cellular immune response (Fig. 10).

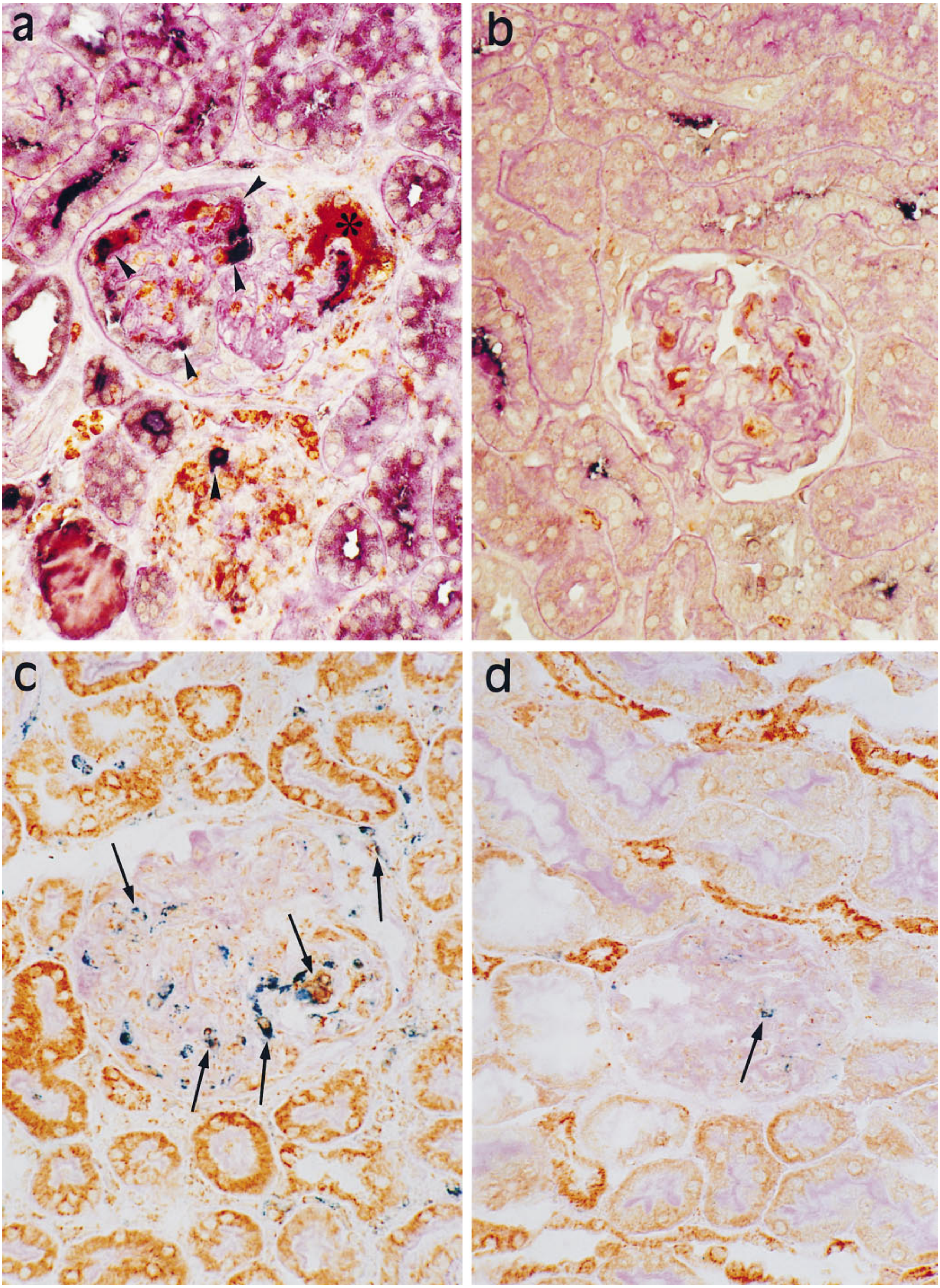
Discussion

This study has shown that administration of a neutralizing antibody to MIF dramatically suppresses an immunologically induced disease model of rapidly progressive crescentic glomerulonephritis. This demonstrates a key regulatory role for MIF in the pathogenesis of immune-mediated glomerulonephritis, a result which may be of great importance to immune-mediated disease in general. The mechanisms by which MIF participates in the disease process are discussed below.

A feature of the study was that while anti-MIF treatment was very effective in inhibiting the progressive phase of rat anti-GBM glomerulonephritis, it had no effect upon the induction of glomerular injury during the first 24 h of the disease. This illustrates an important and previously unrecognized aspect of MIF action. Induction of the early renal injury in this disease model is largely attributed to the early and transient neutrophil influx after the deposition of antibody and complement on the GBM (15, 21, 22). In contrast, the subsequent progression of renal injury is mediated by macrophages and T cells (15–17, 21–27). Therefore, we conclude that MIF is a key regulator of immune disease mediated by macrophages and T cells, but does not participate in neutrophil-mediated tissue injury. It is interesting that the initial phase of glomerular injury in this disease model is also unaffected by blocking the action of the classical proinflammatory cytokines, such as IL-1 (16, 17, 21).

Upregulation of proinflammatory cytokines such as IL-1 may be a key mechanism by which MIF participates in the

Figure 6. Effect of anti-MIF treatment on IL-1β and iNOS expression in rat anti-GBM disease. Double immunohistochemical staining illustrates the following: (*a*) marked upregulation of IL-1β protein by both intrinsic glomerular and tubulointerstitial cells (*dark blue*) and infiltrating ED1$^+$ macrophages (*purple*; *arrow heads*) including multinucleated giant cells (asterisk) on day 14 of control antibody-treated anti-GBM disease; (*b*) a substantial reduction in glomerular, and particularly tubular and macrophage, IL-1β protein by anti-MIF antibody treatment; (*c*) a dramatic increase in both glomerular and tubular iNOS expression (*brown*), including iNOS expression by ED1$^+$ macrophages (*purple*; *arrows*) on day 14 of control antibody-treated anti-GBM disease; and (*d*), a marked inhibition of iNOS expression by anti-MIF antibody treatment. The photomicrographs from the anti-MIF–treated animals represent the ares of the kidney with least inflammatory involvement. Sections were counterstained with PAS. Magnification, ×320.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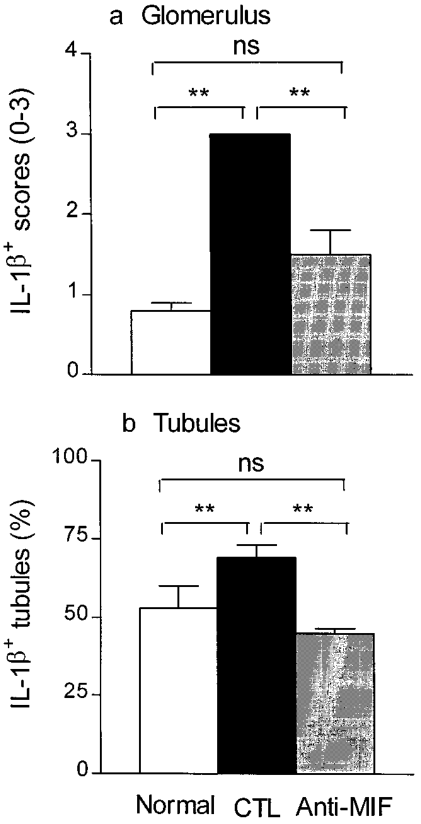

Figure 7. Quantitation of IL-1β expression in rat anti-GBM disease. (*a*) Glomerulus, (*b*) tubules. Anti-MIF treatment almost completely abrogated the upregulation of IL-1β expression seen in control-treated animals. Normal rat kidney (*open bars*), control-treated (*CTL, closed bars*), and anti-MIF–treated animals (*gray bars*). Data is shown as the mean ± SEM for six animals. ns, no significant difference, **$P < 0.01$.

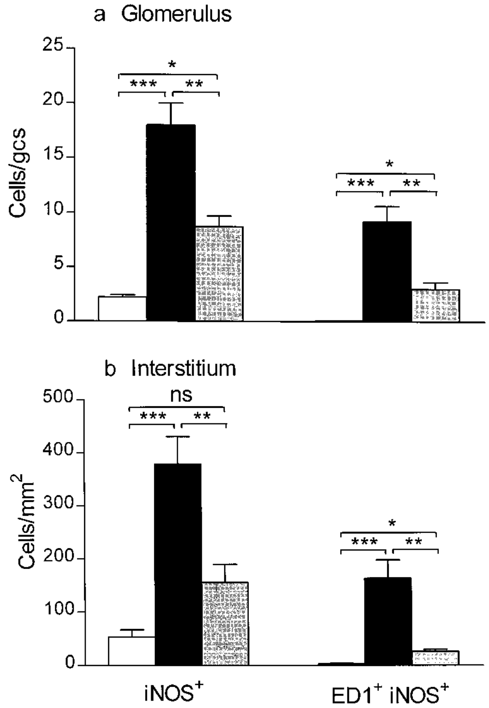

Figure 8. Quantitation of iNOS expression in rat anti-GBM disease. Anti-MIF treatment causes a marked inhibition of iNOS expression by both intrinsic glomerular and interstitial cells and macrophages. (*a*) Glomerulus, (*b*) interstitium. Normal rat kidney (*open bars*), control-treated (*closed bars*) and anti-MIF–treated animals (*gray bars*). Data is shown as the mean ± SEM for six animals. ns, no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

pathogenesis of immunologically mediated renal diseases. There was marked upregulation of IL-1β expression by both intrinsic kidney cells and activated macrophages in rat anti-GBM glomerulonephritis, and previous studies have identified an important role for IL-1 in renal injury in this disease model (16, 17, 21). In vitro, it has been shown that MIF induces IL-1 production by macrophages (5), although IL-1 does not induce macrophage MIF production (28). The ability of anti-MIF treatment to suppress the upregulation of IL-1β expression by both intrinsic kidney cells and infiltrating macrophages in this disease model provides in vivo evidence to support MIF as a physiological inducer of IL-1 production. Therefore, inhibition of disease progression in anti-GBM glomerulonephritis by anti-MIF treatment may be, at least in part, attributed to the inhibition of renal IL-1β expression, because IL-1 receptor antagonist (IL-1Ra) administration has been shown to suppress progressive renal injury in this disease model (16, 17). Furthermore, IL-1 is a potent inducer of ICAM-1 and VCAM-1 expression. Upregulation of ICAM-1 has been shown to be important in the recruitment of leukocytic infiltration in anti-GBM glomerulonephritis (29–31). Treatment of this disease model with IL-1Ra has been shown to significantly suppress ICAM-1 expression and inhibit glomerular and interstitial leukocytic infiltration (32). The ability of anti-MIF mAb in the inhibition of IL-1β expression could be one mechanism by which anti-MIF treatment suppresses ICAM-1 and VCAM-1 expression in this disease, although direct upregulation of leukocyte adhesion molecules by MIF remains unknown.

It has been shown that MIF is a potent inducer of iNOS expression by macrophages in vitro (33, 34). A marked inhibition of iNOS expression by both intrinsic kidney cells and activated macrophages with neutralizing MIF mAb demonstrates that MIF is a key mediator in regulating iNOS expression in anti-GBM glomerulonephritis.

There was substantial leukocyte activation in the damaged kidney in anti-GBM glomerulonephritis, as shown by the presence of many IL-2R–positive cells and macrophages expressing iNOS. Anti-MIF treatment not only significantly reduced leukocytic infiltration in this disease, but also reduced macrophage and T cell immune activation. For example, IL-2R expression, used as a marker of T cell activation, accounted for 46% of interstitial T cells in control antibody-treated disease and this was reduced to 26% of the T cell infiltrate in anti-MIF–treated animals. These results are consistent with the recent finding that MIF plays an important regulatory role in T cell activation, IL-2 production, and proliferation (6).

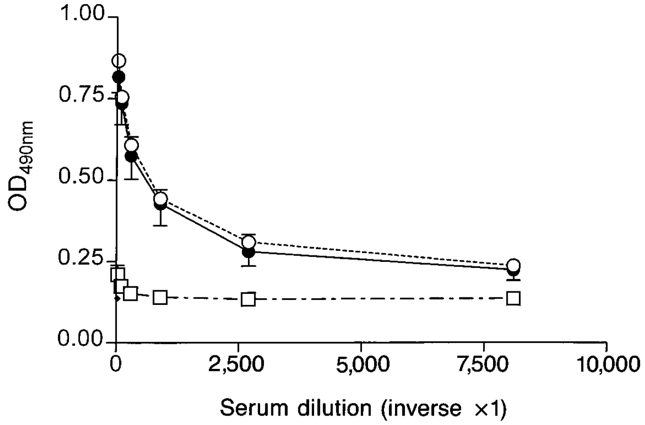

Figure 9. Effect of anti-MIF antibody treatment on the humoral immune response. ELISA analysis shows that there is no different in serum levels of specific rat anti–rabbit IgG between isotype control-treated (*closed circles*; *solid line*) and anti-MIF–treated (*open circles*; *dotted line*) animals. Normal serum IgG levels (*open square*; *dashed line*). Data is shown as the mean ± SEM for six animals.

It has recently been shown that MIF is a key mediator participating in the classical skin DTH response (3). The ability of anti-MIF antibody treatment to inhibit both the skin DTH response to rabbit IgG and renal damage within the kidney argues that similar mechanisms are operating in both tissues. There were strong parallels in the inhibition of leukocyte infiltration, leukocyte activation, and the induction of iNOS expression at both sites. This extends previous studies showing that DTH-like mechanisms operate in rat anti-GBM glomerulonephritis (24).

An interesting observation was that anti-MIF mAb treatment did not affect the humoral immune response in this disease model as measured by serum antibody levels reactive with the immunizing antigen (rabbit IgG). Previous studies have shown that anti-MIF mAb administration can suppress the primary antibody response to RNase A. This study has extended these observations by demonstrating that MIF is not a regulator of the secondary antibody response. This is further supported by the finding that anti-MIF treatment did not alter the immune deposition with the kidney. Thus, the ability of anti-MIF mAb treatment to suppress anti-GBM glomerulonephritis was solely due to its inhibition of the cell-based mechanisms of tissue injury.

Finally, an interesting issue was the effect of anti-MIF treatment on the marked upregulation of renal cortical MIF expression seen in this disease model. It has been found that there was marked de novo MIF expression by intrinsic kidney cells including glomerular and tubular epithelial cells, endothelium, interstitial fibroblasts in anti-GBM glomerulonephritis, and upregulation of renal MIF correlates with glomerular and tubulointerstitial macrophage accumulation and progressive renal injury including glomerular crescent formation (7). In the present study, functional blocking of MIF activity with the neutralizing MIF mAb resulted in a substantial inhibition of MIF mRNA and protein expression by both intrinsic kidney cells and infiltrating macrophages. This raises the possibility that MIF produced either locally or systemically may act, as an autocrine, to amplify its own production during the inflammatory/immune response.

In conclusion, this study has demonstrated a key regulatory role for MIF in the pathogenesis of immunologically induced kidney disease. Not only does this argue that blocking MIF activity may be of benefit in the treatment of human rapidly progressive glomerulonephritis, but strategies to block the action of MIF may be important in immune-mediated disease generally.

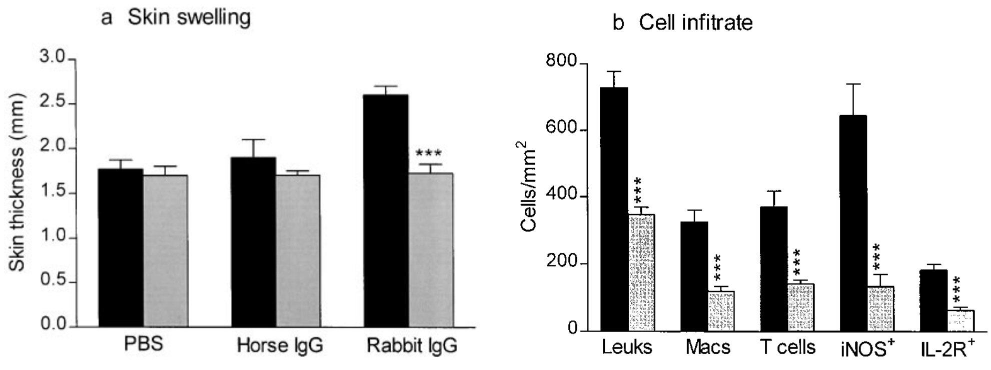

Figure 10. Effect of anti-MIF antibody treatment on the skin DTH response. (*a*) Skin swelling, (*b*) leukocytic infiltration and activation. Anti-MIF treatment (*gray bars*) abrogated the skin swelling and macrophage and T cell accumulation and activation (iNOS and IL-2R expression) in the skin DTH response to specific challenge rabbit IgG. Data is shown as the mean ± SEM for six animals. ***$P < 0.001$ versus control (*closed bars*) antibody treated by an unpaired two-sided Student's *t*-test.

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