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Role of interleukin-6 in mediating mesangial cell proliferation and matrix production *in vivo*

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Role of interleukin-6 in mediating mesangial cell proliferation and matrix production *in vivo*. Mesangial cell proliferation and matrix overproduction characterize many progressive glomerular diseases. Based on currently available data, the role of interleukin-6 (IL-6) in mediating mesangial cell proliferation and matrix production is controversial. The present study attempts to clarify this issue by showing that: (1) IL-6 knock out mice develop a normal glomerular architecture and in particular a normal mesangium. (2) Mesangioproliferative glomerulonephritis induced by Habu snake venom is equally severe in IL-6 knock out mice as in control mice. (3) A continuous seven-day intraperitoneal infusion of 50 μg recombinant human IL-6 into rats with a prior minimal (subnephritogenic) injury to mesangial cells does not induce glomerular cell activation, cell proliferation, matrix production, leukocyte influx, platelet influx or proteinuria. (4) A continuous seven-day IL-6 infusion into rats with mesangioproliferative nephritis (anti-Thy 1.1 nephritis) increases matrix protein transcription in the absence of detectable effects on matrix protein accumulation and otherwise has no effect on the natural course of the disease. We conclude from these findings that IL-6 is not an important mediator of mesangial cell proliferation and matrix overproduction *in vivo*, and that currently little rationale exists to advocate anti-IL-6 therapy in mesangioproliferative disease states.

Mesangial cell proliferation and mesangial matrix accumulation are key features of various human glomerular diseases, including IgA nephropathy, non-IgA mesangioproliferative glomerulonephritis, membranoproliferative glomerulonephritis, variants of idiopathic focal sclerosis, lupus nephritis, and possibly diabetic nephropathy [1, 2]. Mesangial cell proliferation and glomerular matrix accumulation may also contribute to the development of glomerulosclerosis independent of the underlying primary disease [3, 4]. Therefore, the study of factors that drive mesangial cell proliferation and matrix production is important to understand the pathogenesis and to design new therapeutic approaches for mesangioproliferative glomerulonephritis and progressive glomerulosclerosis.

Interleukin-6 (IL-6) is produced in relatively large amounts by mesangial cells in response to a variety of stimuli, such as angiotensin II, lectins, matrix proteins, cytokines, and immune complexes [5–11]. IL-6 has been reported to induce matrix protein transcription and autocrine growth in mesangial cells *in vitro* [12–14]. *In vivo*, glomerular IL-6 overexpression was detected in human glomerulonephritis types characterized by mesangial hypercellularity such as IgA nephropathy and some types of lupus nephritis [15–19]. Furthermore, IL-6 transgenic mice developed a mesangioproliferative glomerulonephritis [13] and the urinary excretion of IL-6 has been correlated with mesangial hypercellularity in patients with IgA nephropathy [13, 20, 21]. It has therefore repeatedly been proposed that IL-6 is an important mediator of mesangial cell proliferation and matrix overproduction [2, 8, 16, 22]. This theory has been challenged by several observations. First, IL-6 transgenic mice develop a massive polyclonal B-cell activation, which in itself may be associated with mesangioproliferative disease [23]. Second, the role of IL-6 in mediating mesangial cell proliferation *in vitro* is controversial, with follow-up studies showing either no effect [24, 25] or even growth inhibition of mesangial cells treated with IL-6 [26]. Third, increased urinary IL-6 excretion has been detected in a variety of renal abnormalities and several patients with mesangial hypercellularity failed to excrete detectable urinary IL-6 [21, 27].

In the present study we have attempted to further define the role of IL-6 in the mediation of mesangial cell proliferation and matrix production *in vivo*. Four experimental approaches were chosen: (1) determine whether IL-6 knock out mice develop a normal mesangium; (2) investigate whether experimental mesangioproliferative glomerulonephritis can be generated in IL-6 knock-out mice in a similar manner as in control mice; (3) investigate the effects of an IL-6 infusion in rats with a prior minimal (subnephritogenic) injury to the mesangial cells; (4) investigate the effect of IL-6 infusion in rats with mesangioproliferative nephritis (anti-Thy 1.1 nephritis).

Methods

Experimental design

All animal experiments were approved by the local review boards.

Glomerular morphology in IL-6 knock-out mice

Kidneys were obtained from 6 homozygous IL-6 knock-out mice [28] (bred in the animal facilities of the Hannover Medical School, 3 males, 3 females, age 58 to 60 days), 2 heterozygous IL-6 knock-out/wild-type mice (2 females, age 58 days) and 6 wild-type

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control mice (strain C57BL/6, obtained from the Zentralinstitut für Versuchstierkunde, Hannover Medical School; 4 males, 2 females, age 54 days). Prior to sacrifice, a 24-hour urine collection was performed and a serum sample was collected. The IL-6 genotype of each IL-6 knock-out mouse was verified by Southern blot analysis.

Mesangioproliferative glomerulonephritis in IL-6 knock-out mice

Mesangioproliferative glomerulonephritis was induced in 6 homozygous IL-6 knock-out mice (3 males, 3 females, age 90 days) and 6 wild-type control mice (3 males, 3 females, age 85 days) by a single intravenous bolus injection of 4 mg/kg body wt Habu snake venom (Trimeresurus flavoviridis; Sigma, Deisenhofen, Germany). Following the injection, all mice were kept under an infrared light for the next three hours to reduce mortality. A 24-hour urine collection was performed from days 5 to 6 after disease induction. Mice were sacrificed at day 6 for the histological examination of the kidneys.

IL-6 infusion in rats following injection of a subnephritogenic anti-Thy 1.1 dose

Eleven male Wistar rats (Charles River) weighing about 180 g received an intravenous bolus injection of 0.2 mg/kg monoclonal anti-Thy 1.1 IgG_1 (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK). Microosmotic pumps (filling volume 200 μl, delivery rate 1 μl/hr; Alzet Corporation, Palo Alto, CA, USA) were then loaded with 50 μg recombinant human IL-6 ($N = 5$; kindly supplied by Dr. Amando Proudfood, Glaxo Institute for Molecular Biology, Geneva, Switzerland) or phosphate buffered saline (PBS; $N = 6$). The pumps were implanted into the peritoneal cavity at four hours after disease induction to avoid any interference with glomerular anti-Thy 1.1 antibody binding, which is maximal at one hour after injection [29]. One hundred microliters of citrate-anticoagulated plasma samples were collected at days 0, 2 and 7 after disease induction and 24 hour urine was collected from days 6 to day 7. Renal biopsies for histological examination of the kidneys were obtained at days 2, 4 and 7 (sacrifice) after disease induction. Prior to the day 2 renal biopsy, proliferating cells had been labeled with 5-bromo-2′-deoxyuridine (BrdU; Sigma) by an intraperitoneal injection of 100 mg/kg body wt at 24, 32, and 40 hours after disease induction. Following the renal biopsies at day 7, glomeruli were isolated from the remaining pooled renal cortices of all rats per group by differential sieving and glomerular RNA was prepared.

IL-6 infusion in rats with anti-Thy 1.1 mesangioproliferative glomerulonephritis

Eleven male Wistar rats (Charles River) weighing about 180 g received an intravenous bolus injection of 1 mg/kg monoclonal anti-Thy 1.1 IgG_1 (clone OX-7). Fourty-eight hours later, that is, after the peak of mesangiolysis and at the start of the mesangioproliferative phase, a microosmotic pump (filling volume 200 μl, delivery rate 1 μl/hr) was implanted into the peritoneal cavity. Pumps were loaded with 50 μg recombinant human IL-6 ($N = 5$) or PBS ($N = 6$). One hundred microliter plasma samples were collected at days 2, 4 and 9 after disease induction and 24 hour urine was collected from days 8 to 9. Renal biopsies for histological evaluation were obtained at days 4, 6 and at sacrifice (day 9). Prior to the day 6 renal biopsy, proliferating cells had been labeled with BrdU by an intraperitoneal injection at 120, 128, and 136 hours after disease induction. At sacrifice (day 9) glomeruli were isolated by differential sieving from the pooled renal cortices of all rats per group and glomerular RNA was prepared.

Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [30] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. In the PAS stained sections the total number of cells per glomerular cross section as well as the number of mitoses within the glomerular tuft (extrapolated to mitoses per 100 glomerular cross sections) was determined.

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by a direct or indirect immunoperoxidase technique as previously described [30]. Primary antibodies included:

- 1A4, a murine monoclonal antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin (Dako, Glostrup, Denmark) [31].
- D33, a murine monoclonal IgG_1 antibody against human muscle desmin (Dako) [32].
- PC 10 (Oncogene Science Inc., Uniondale, NY, USA), a murine IgM monoclonal antibody against human PCNA, which is expressed by actively proliferating cells. We have previously shown, in angiotensin II infused rats, that cell proliferation as assessed by anti-PCNA antibody correlates with the cell proliferation as assessed by the conventional method of ^{3}H-thymidine incorporation [33].
- BU-1, a murine monoclonal antibody against bromo-deoxyuridine [34] containing nuclease in Tris buffered saline (Amersham, Braunschweig, Germany).
- ED1 (Serotec, Oxford, UK), a murine monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells [35].
- PL-1, a murine monoclonal antibody against rat platelets [36].
- affinity purified polyclonal goat anti-human/bovine type IV collagen (Southern Biotechnology Inc., Birmingham, AL, USA).
- an affinity purified IgG fraction of polyclonal rabbit anti-rat fibronectin (Southern Biotechnology).
- a polyclonal, biotinylated horse anti-mouse IgG antibody (Vector, Burlingame, CA, USA).

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit or goat IgG. Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

To obtain mean numbers of proliferating cells or infiltrating leukocytes in glomeruli, more than 30 consecutive cross sections of glomeruli containing more than 20 discrete capillary segments were evaluated and mean values per kidney were calculated. To obtain total counts of proliferating cells or infiltrating leukocytes in the renal cortical tubulointerstitium over 40 grid fields (range 40 to 60), measuring 0.36 mm^2 each, were analyzed and, again, mean counts per kidney were obtained. For the evaluation of the immunoperoxidase stains for α-smooth muscle actin, desmin, type IV collagen, fibronectin and platelets, each glomerular area and

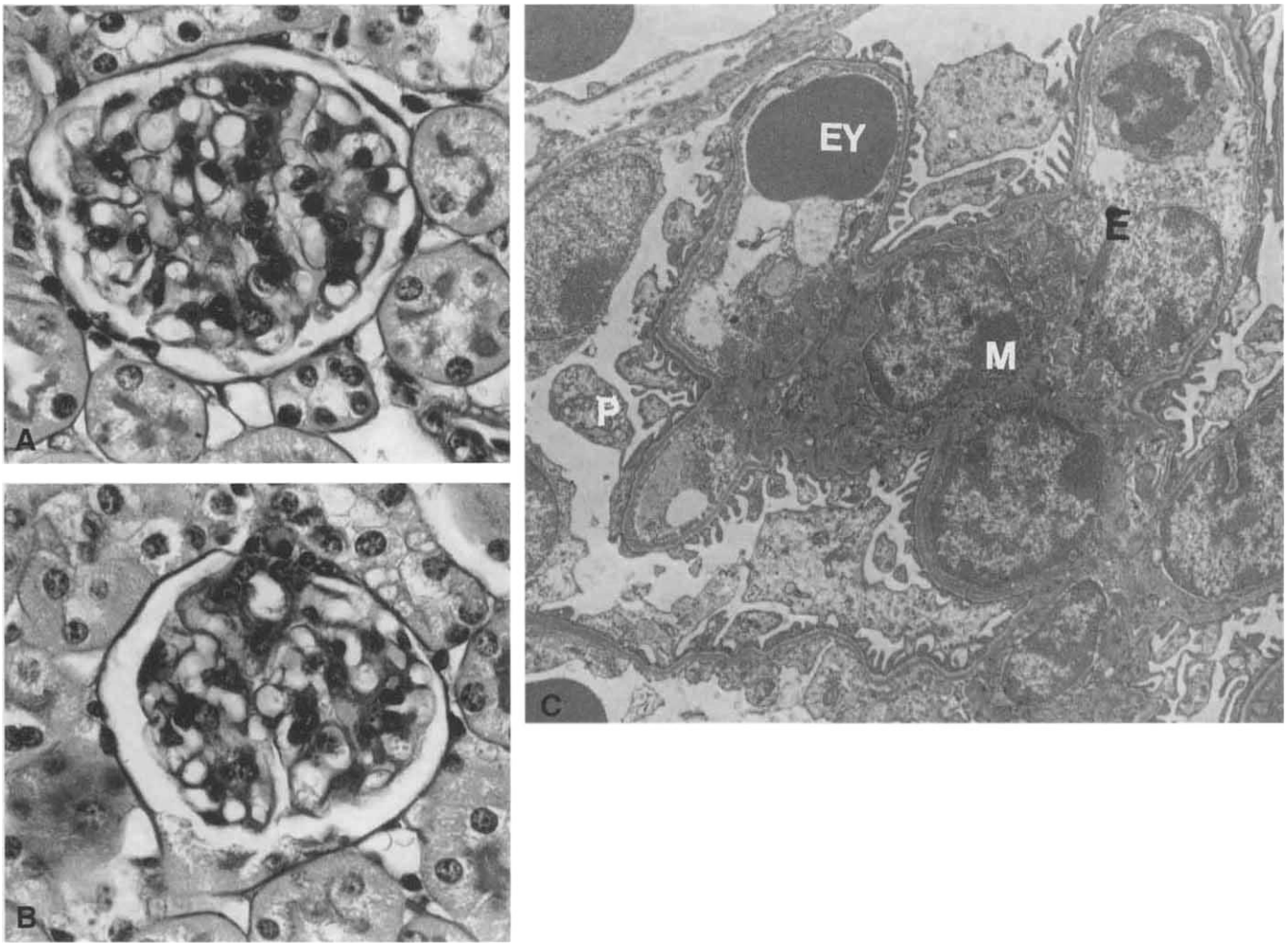

Fig. 1. *Light microscopic appearance of a typical glomerulus of an IL-6 knock-out mouse (***A***) and a wild-type mouse (***B***).* No abnormal morphological features are present (PAS stain; magnification × 1000). C and D show the electron microscopic appearance of glomerular segments of IL-6 knock-out mice. (**C**) Glomerular segment with regular mesangium, typical mesangial cells and no increase in mesangial matrix; the glomerular basement membrane and podocytes also exhibit a normal morphology. (**D**) Higher magnification of a mesangial cell with a typical nucleus and typical filaments at the plasma membrane. Abbreviations are: M, mesangial cell; E, endothelium; EY, erythrocyte; P, podocyte. Light microscopic appearance of a glomerulus with a mesangial nodule in an IL-6 knock-out mouse (**E**) or wild-type mouse (**F**) at six days after the injection of Habu snake venom. Nodule formation appears similar in both mouse strains. (PAS stain; magnification × 1000).

tubulointerstitial grid field was graded semiquantitatively, and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of staining and depended on the percentage of the glomerular tuft area or grid field showing positive staining: 0 = absent staining or less than 5% of the area stained; I = 5 to 25%; II = 25 to 50%; III = 50 to 75%; IV = >75%. We have recently described that this semiquantitative scoring system is not only highly reproducible among different observers, but that the data also are highly correlated with those obtained by computerized morphometry [37].

Staining for glomerular mouse IgG, that is, anti-Thy 1.1 antibody, was graded as 0 (negative), I (trace), II (moderate) or III (strong), and a mean glomerular score was calculated.

Electron microscopy

Renal tissue was cut into 1 mm slices and fixed for 24 hours in a 2.5% glutaraldehyde solution. Tissue was then embedded in araldite. Ultrathin sections were contrasted with lead citrate and viewed in a transmission electron microscope (Zeiss EM900).

Preparation of glomerular RNA and Northern analysis

Glomeruli were isolated by differential sieving [38]. All glomerular isolates were checked microscopically and exhibited a purity of greater than 98%. Total RNA was extracted from glomeruli with guanidinium isothiocyanate and subsequent ultracentrifugation through caesium chloride using standard procedures [39, 40]. The RNA content of the samples obtained was determined by UV spectrophotometry at 260 and 280 nm. Samples with an OD 260/280 nm ratio of < 1.8 were discarded. For Northern analysis the RNA was denatured and 3 or 10 μg/lane were electrophoresed through a denaturing 1% agarose/formaldehyde gel [41]. Integrity of the RNA was assessed by visualization of the 28S and 18S rRNA bands. Separated RNA was then transferred onto a nylon membrane (Hybond N^{+}; Amersham, Braunschweig, Germany) by

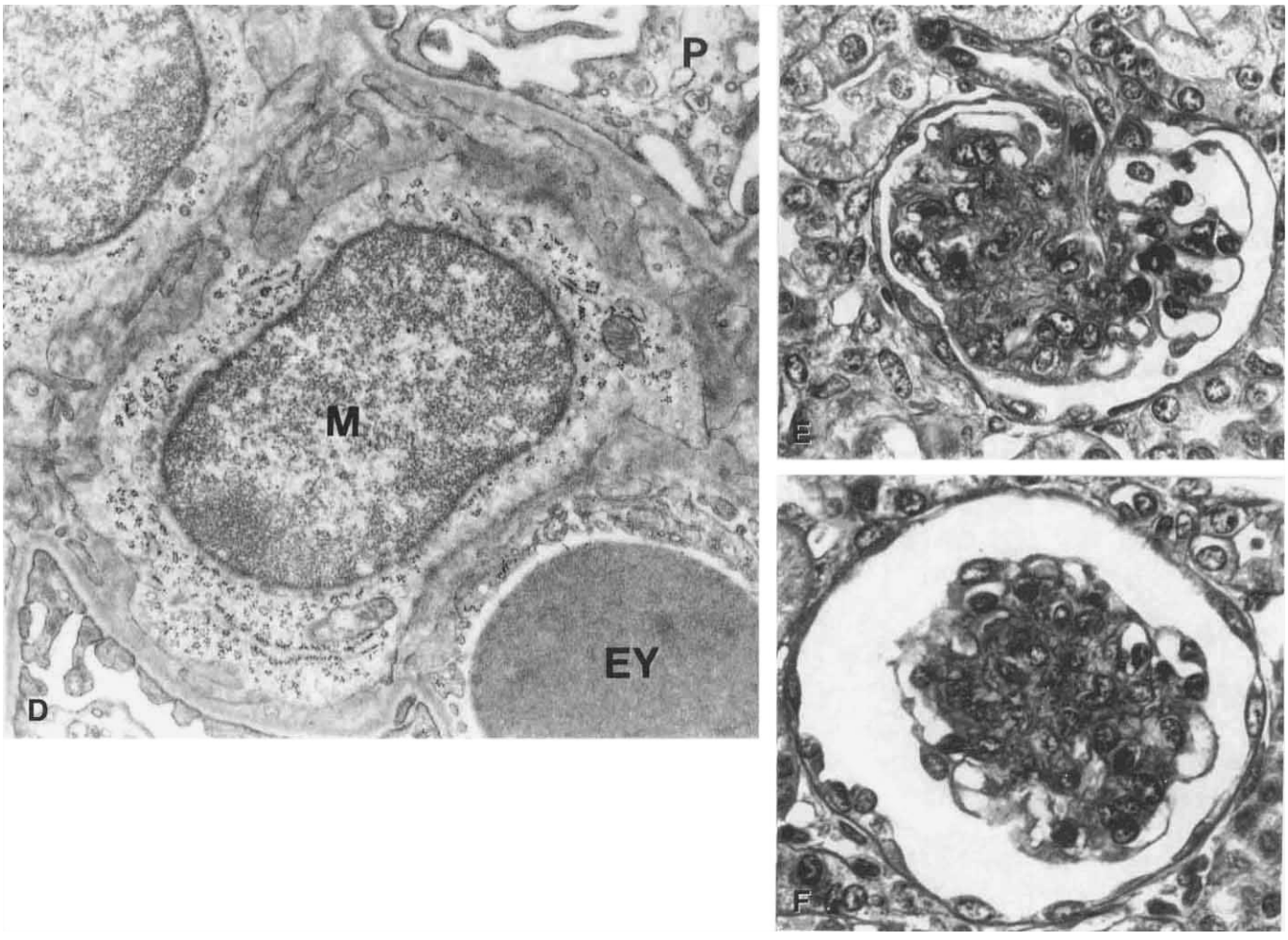

Fig. 1. *Continued.*

capillary blotting and cross linked using a UV-crosslinker (Stratagene, Heidelberg, Germany). Hybridization was performed using digoxigenin labeled riboprobes which were generated using a Digoxigenin RNA Labeling Kit (Boehringer, Mannheim, Germany) and hybridized probe was detected using the Digoxigenin Nucleic Acid Detection Kit (Boehringer) with minor modifications. Band intensities were scanned with a densitometer (Herolab, Wiesloch, Germany) and corrected for the relative intensities of the 28S rRNA signal as detected by hybridization with a 28S rRNA cDNA probe.

The fibronectin and α_1(IV) collagen probe were 420 bp and 296 bp, respectively, cDNAs which were generated from total rat glomerular RNA by RT PCR. The sequence of the primers were: 5′-CGTGAATTCCAGGCACTGACTACAAGATC-3′ (fibronectin sense); 5′-CGGTCACTCGAGCGATGACATAGATGGTGTAC-3′ (fibronectin antisense); 5′-CGTGAATTCGTGCGGTTTGTGAAGCACCG-3′ (α_1(IV) collagen sense); and 5′-AGCTCACTCGAGCTTCTTGAACATCTCGCTT-3′ (α_1(IV) collagen antisense).

For *in vitro* transcription, the PCR products were cloned into pBluescipt (Stratagene).

The 28S rRNA cDNA (a gift of L. Iruela-Arispe and H. Sage) [42] was labeled with digoxigenin by random priming using a digoxigenin random priming kit (Boehringer Mannheim) according to the manufacturer's instructions.

Monitoring of the IL-6 infusion

To examine whether the dosage regimen was appropriate, the infused human recombinant IL-6 and the biological effects were assessed by measuring plasma concentrations of α_2-macroglobulin (the major hepatic acute phase protein in rats) by immunoelectrophoresis as described [43]. IL-6 concentrations were measured in the plasma samples using a commercially available kit (R&D Systems, Biermann, Bad Nauheim, Germany). Finally, all microosmotic pumps were explanted at the end of the infusion period (day 7) and cut open to ensure that all IL-6 had been delivered from the pumps.

Miscellaneous measurements

Urinary protein was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard. Creatinine and urea were measured in serum using an

Table 1. Glomerular characteristics of non-manipulated, 54- to 60-day-old homozygous or heterozygous IL-6 knock-out or wild-type mice

	IL-6 knock-out mice (homozygous) (N = 6)	IL-6 knock-out mice (heterozygous) (N = 2)	Wild-type mice (N = 6)
Number of nuclei per glomerular cross section	35 ± 13	40/33	36 ± 14
Glomerular α-smooth muscle actin staining score	0.20 ± 0.03	0.20/0.15	0.18 ± 0.04
Glomerular desmin staining score	1.02 ± 0.03	1.11/1.00	1.03 ± 0.04

Data are mean ± SD or individual values (heterozygous mice).

Table 2. Characteristics of IL-6 knock-out or wild-type mice at 6 days after the induction of Habu snake venom mesangioproliferative glomerulonephritis

	IL-6 knock-out mice (N = 6)	Wild-type mice (N = 6)	P
Hypercellular glomeruli	20.5 ± 13.7%	22.2 ± 21.0%	NS
Glomeruli with mesangial nodule formation[a]	0;0;0;0;10;13%	0;0;0;0;2;45%	NS
Glomerular α-smooth muscle actin staining score	0.33 ± 0.07	0.63 ± 0.77	NS
Glomerular desmin staining score	1.44 ± 0.27	1.65 ± 0.70	NS
Proteinuria *mg/24 h*	3.9 ± 1.9	3.1 ± 1.8	NS

NS is not significantly different.
[a] Individual frequencies per animal are shown

autoanalyzer (Beckman Instruments GmbH, München, Germany).

Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using the Mann-Whitney rank sum test. Correlations were assessed using linear regression analysis.

Results

IL-6 knock-out mice develop a normal mesangium

As shown in Figure 1 A and B, IL-6 knock-out mice displayed a glomerular and mesangial architecture that did not differ from that of wild-type mice. Total glomerular nucleus counts in homozygous and heterozygous IL-6 knock-out mice were not different from those obtained in wild-type mice (Table 1). Similarly, glomerular expression of α-smooth muscle actin, a marker of activated mesangial cells [38], was equally absent in all three groups, while desmin, which is constitutively expressed by mesangial cells [38], was equally present in all three groups (Table 1). By electron microscopy glomeruli showed a regularly developed mesangium with a normal cell number. The glomerular basement membrane was typically structured and covered by podocytes with typical foot processes (Fig. 1 C, D). No significant proteinuria or hematuria was present in homozygous or heterozygous IL-6 knock-out mice, and serum urea and creatinine values were within the normal range in all mice (data not shown).

Mesangioproliferative glomerulonephritis similarly develops in IL-6 knock-out and wild-type mice

In pilot experiments 4 mg/kg Habu snake venom was identified as the maximally tolerated dose, since at 5 mg/kg of the venom a high mortality was observed. At the 4 mg/kg dose about 20% of the glomeruli exhibited hypercellular areas in both IL-6 knock-out and wild-type mice at day 6 after disease induction (Table 2). In each group nodular mesangial lesions (Fig. 1 E, F) developed in 2 of 6 rats. The frequency of mesangial nodules did not differ significantly between IL-6 knock-out and wild-type mice (Table 2). Furthermore, scoring of glomerular markers of mesangial cell activation, namely the expression of desmin and the *de novo* expression of α-smooth muscle actin, did not reveal significant differences between the two groups (Table 2). Neither IL-6 knock-out nor wild-type mice developed significant proteinuria (Table 2).

IL-6 infusion has no effects in rats with preceding minimal mesangial injury

In PBS infused rats the injection of a low dose of OX-7 anti-Thy 1.1 antibody led to mild glomerular *de novo* α-smooth muscle actin expression, a minor increase in desmin expression, as well as a low grade increase in the number of glomerular mitotic figures, monocyte/macrophage influx and matrix protein accumulation, while no significant platelet influx was observed (Fig. 2). Cell proliferation as assessed by counting of glomerular mitoses was correlated with the counts of PCNA-positive nuclei ($r = 0.75$, $P < 0.001$) as well as with counts of BrdU positive nuclei ($r = 0.56$, $P = 0.07$). None of the aforementioned parameters was significantly altered in IL-6 infused rats as compared to PBS infused rats (Fig. 2). Northern analysis of glomerular RNA showed no detectable expression of fibronectin mRNA in both groups under our study conditions (data not shown), while α_1(IV) collagen mRNA was weakly expressed in both IL-6 and PBS infused rats (Fig. 3A). Densitometry and correction for the expression of 28S rRNA yielded an 1.4-fold (range 1.1- to 1.7-fold; $N = 3$) increase of α_1(IV) collagen mRNA in IL-6 infused rats as compared to PBS infused rats. In neither group did significant proteinuria or hematuria develop. Weight gain during the seven-day study period was similar (PBS infused rats, 5 ± 8 g; IL-6 infused rats, 7 ± 8 g). IL-6 infusion also had no apparent influence on the binding of anti-Thy 1.1 antibody in the mesangium since staining for murine IgG was not significantly different from that observed in PBS infused rats (Fig. 2).

No tubulointerstitial damage occurred in PBS and IL-6 infused rats as judged by PAS-staining of the sections and by the absence of interstitial α-smooth muscle actin and desmin expression, normal PCNA counts, monocyte/macrophage counts and normal staining for type IV collagen and fibronectin (data not shown).

Table 3 shows that the infused IL-6 was biologically active, since at day 2 of the infusion period plasma concentrations of the acute phase protein α_2-macroglobulin were significantly elevated over those observed in PBS infused rats. Comparable levels of plasma α_2-macroglobulin have been measured previously after a bolus injection of 4 μg IL-6 or 0.25 μg lipopolysaccharide into normal rats [43]. Similar to findings in nephrotoxic nephritis [43], the induction of immune-mediated glomerular injury also induced to some degree the acute phase response, since α_2-macroglobulin

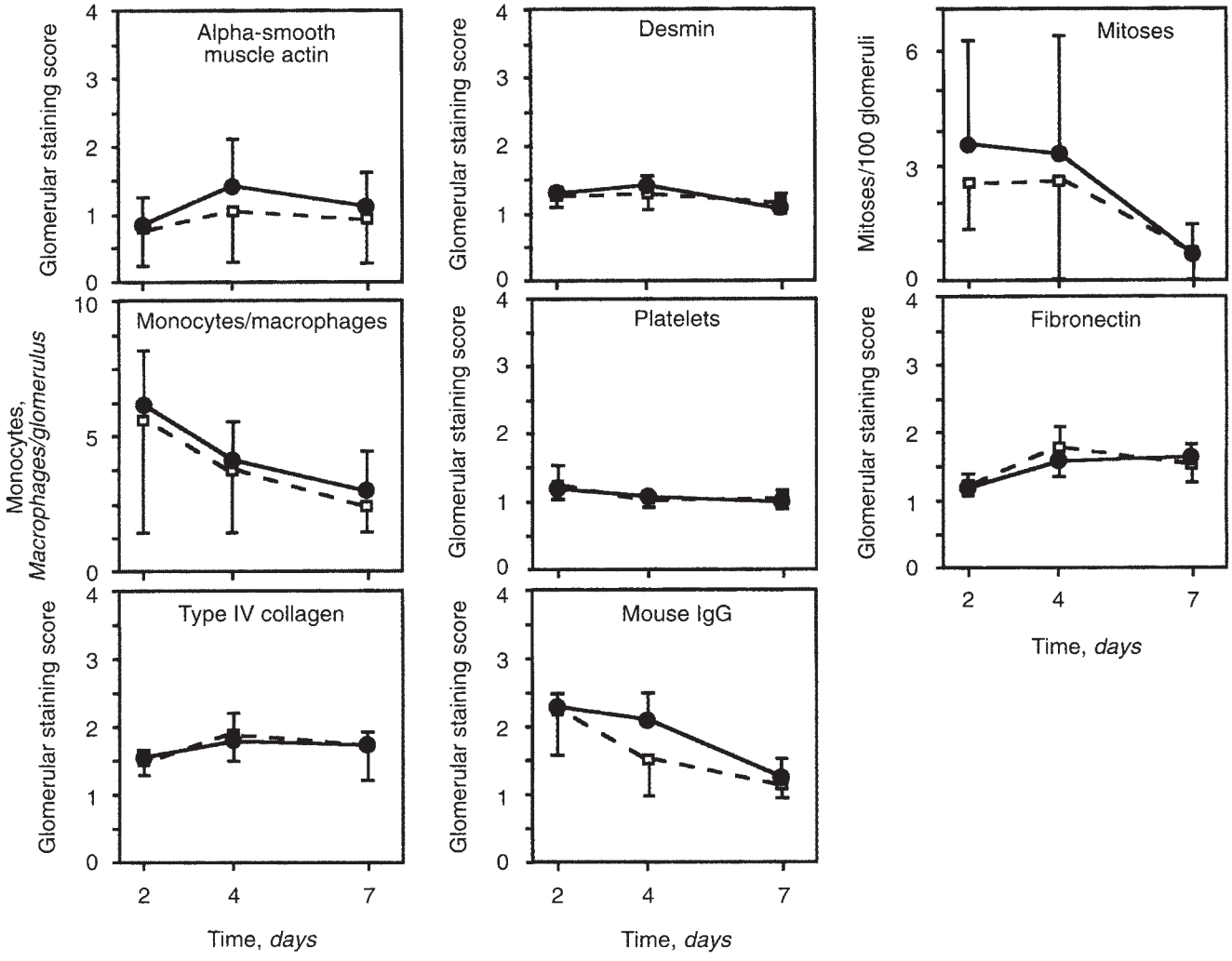

Fig. 2. *Glomerular changes in rats with subnephritogenic mesangial injury* (**Methods**) *infused from days 0 to 7 with either PBS (□; N = 6) or 50 μg recombinant human IL-6 (●; N = 5).* Values are means ± SD.

concentrations increased with time in the plasma of PBS infused rats (Table 3). Measurement of the IL-6 plasma concentrations obtained during the infusion period confirmed that high circulating levels were achieved at day 2, and that they dropped rapidly after the end of the infusion, that is, at day 7 (Table 3).

IL-6 infusion induces matrix protein transcription but has no other effects in rats with mesangioproliferative glomerulonephritis

In PBS infused rats the injection of the regular dose of OX-7 anti-Thy 1.1 antibody led to marked glomerular *de novo* α-smooth muscle actin expression, a marked increase in desmin expression, as well as increased cell proliferation, monocyte/macrophage influx, platelet influx and matrix protein accumulation (Fig. 4) similar to previously described findings [30, 38, 44]. Again, none of the aforementioned parameters was significantly altered in IL-6 infused rats as compared to PBS infused rats (Fig. 4). Cell proliferation was also not significantly different between IL-6 and PBS infused rats when assessed by PCNA staining or BrdU incorporation (data not shown). Northern analysis of glomerular RNA for the expression of fibronectin or α_1(IV) collagen mRNA showed an up-regulation of both RNA species in the IL-6 infused rats as compared to PBS infused rats (Fig. 3B). Densitometry and correction for the expression of 28S rRNA yielded 6.0-fold (range 2.2- to 11.2-fold; $N = 3$) increases of fibronectin mRNA in IL-6 infused rats as compared to PBS infused rats, and 2.0-fold (range 1.8- to 2.1-fold; $N = 3$) increases of α_1(IV) collagen mRNA. Proteinuria (54.2 ± 34.4 mg/24 hr) but no hematuria was present at day 8 in PBS infused rats and was not significantly different from that observed in IL-6 infused rats (64.8 ± 26.9 mg/24 hr). Weight gain during the seven-day study period was not significantly different (PBS infused rats, 5 ± 33 g; IL-6 infused rats, 27 ± 8 g). IL-6 infusion also had no apparent influence on the binding or clearance of anti-Thy 1.1 antibody in the mesangium since staining for murine IgG was not significantly different from that observed in PBS infused rats (Fig. 4).

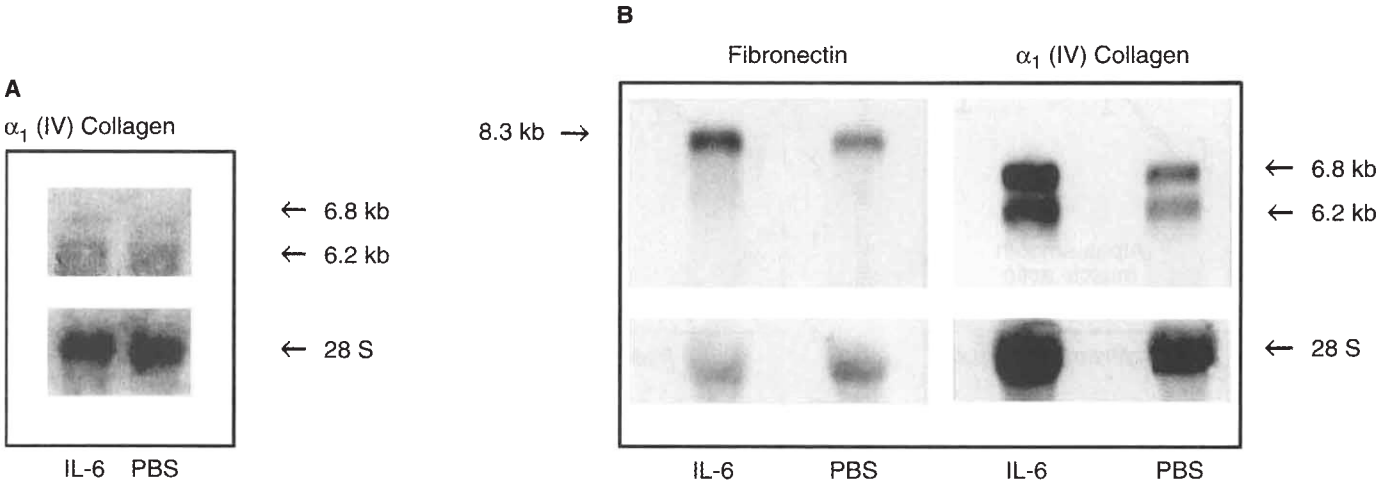

Fig. 3. *(A) Demonstration of α_1(IV) collagen mRNA in total glomerular RNA isolated from rats with subnephritogenic mesangial injury and infused from days 0 to 7 with either PBS or 50 μg recombinant human IL-6.* **(B)** Demonstration of fibronectin and α_1(IV) collagen mRNA in total glomerular RNA isolated from rats with mesangioproliferative anti-Thy 1.1 nephritis infused from days 2 to 9 after disease induction with either PBS or 50 μg recombinant human IL-6.

Table 3. Plasma concentrations of α_2-macroglobulin and IL-6 in IL-6 or PBS infused rats with either subnephritogenic mesangial injury or anti-Thy 1.1 mesangioproliferative glomerulonephritis

	Plasma α_2-macroglobulin concentration *μg/ml*		Plasma IL-6 concentration *pg/ml*	
	IL-6 infused	PBS infused	IL-6 infused	PBS infused
Subnephritogenic anti-Thy 1.1 dose; *day after disease induction*				
day 0	135 ± 33	119 ± 32	0 ± 0	0 ± 0
day 2	5123 ± 1137	1374 ± 699[a]	52 ± 21	0 ± 0[a]
day 7	1678 ± 474	1142 ± 368	5 ± 2	0 ± 0[a]
Nephritogenic anti-Thy 1.1 dose; *day after disease induction*				
day 2	550 ± 158	852 ± 357	0 ± 0	0 ± 0
day 4	3634 ± 1431	1573 ± 407[a]	42 ± 15	3 ± 3[a]
day 9	3744 ± 1835	2636 ± 526	12 ± 10	2 ± 5

[a] $P < 0.05$ versus PBS infused rats

No tubulointerstitial damage occurred in PBS and IL-6 infused nephritic rats as judged by PAS-staining of the sections and by the absence of interstitial α-smooth muscle actin and desmin expression, normal PCNA counts, monocyte/macrophage counts and normal staining for type IV collagen and fibronectin (data not shown).

As in the experiments with subnephritogenic anti-Thy 1.1 antibody doses, Table 3 again confirms that the infused IL-6 was biologically active in the nephritogenic rats and that high circulating levels of IL-6 were achieved during the infusion period.

Discussion

Glomerular morphology in IL-6 knock-out mice

To evaluate the potential role of IL-6 in mediating mesangial cell proliferation and matrix production, we first examined the glomerular morphology of IL-6 knock-out mice. The rationale for this approach is provided by observations that ontogenetic events are frequently recapitulated during glomerular disease [45]. Therefore, mesangial maldevelopment in IL-6 knock-out mice would point to an ontogenetically important role of IL-6 and would imply that IL-6 may play a similarly important role in glomerulonephritis. However, we could not detect any significant difference in the mesangial morphology of IL-6 knock-out mice by light and electron microscopy when compared to wild-type mice. Furthermore, the immunostaining pattern in these mice for two cytoskeletal proteins was normal, namely desmin, whose expression is restricted to normal mesangial cells, and α-smooth muscle actin, which is only expressed by activated mesangial cells *in vivo* [38]. It could be argued that these findings do not exclude an important role of IL-6 in mesangial ontogenesis since other growth factors might have compensated for the lack of IL-6. However, it is noteable that in the case of another well established mesangial cell mitogen, platelet-derived growth factor (PDGF), both PDGF B-chain knock-out mice as well as PDGF β-receptor knock-out mice completely failed to develop a mesangium [46, 47].

Mesangioproliferative glomerulonephritis in IL-6 knock-out mice

To further define the role of IL-6 in regulating mesangial cell behavior *in vivo*, we adapted the rat model of Habu snake venom-induced mesangioproliferative nephritis to mice. Similar to findings in rats [48], Habu snake venom induced a focal glomerulonephritis in wild-type mice, with less than one third of the glomeruli involved. Again, genetic IL-6 deficiency had no effect on the manifestation of the Habu nephritis, suggesting that IL-6 is not of central importance in mediating mesangial cell proliferation and matrix accumulation in this murine model of mesangioproliferative glomerulonephritis.

IL-6 infusion in rats following injection of a subnephritogenic anti-Thy 1.1 dose

In normal rats, toxicity studies performed by others using very high IL-6 doses (daily subcutaneous injections of up to 500 μg/kg

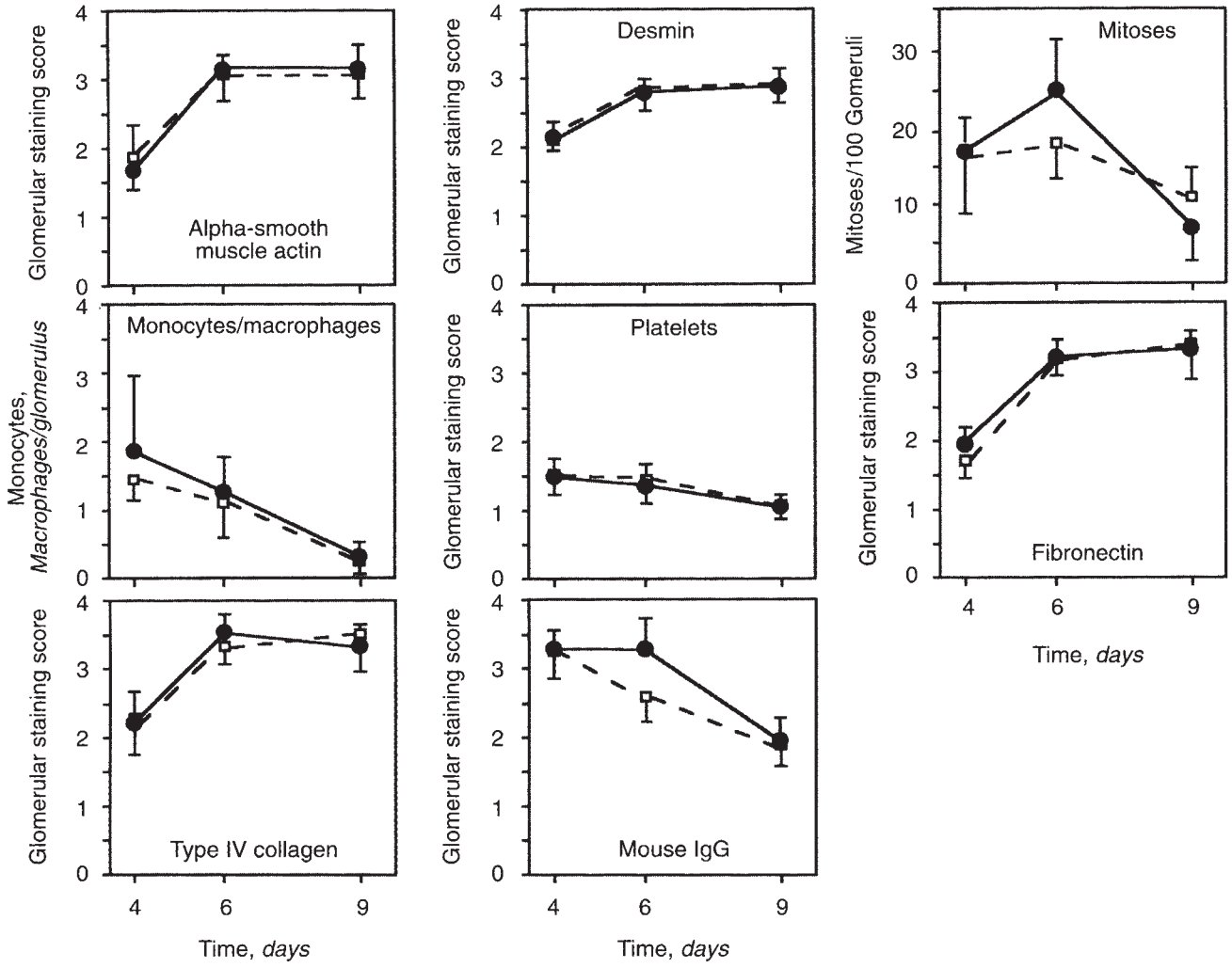

Fig. 4. *Glomerular changes in rats with mesangioproliferative anti-Thy 1.1 nephritis infused from days 2 to 9 after disease induction with either PBS (□; N = 6) or 50 μg recombinant human IL-6 (●; N = 5).* Values are means ± SD.

for 30 days) have failed to induce any renal abnormalities in rats or mice [49, 50]. However, failure to detect a cytokine effect in normal animals does not exclude a potential role in disease. Hence, we have described that other mesangial cell growth factors, including basic fibroblast growth factor (bFGF) and PDGF also exhibited no or little proliferative activity in rat glomeruli when infused into normal rats [51–53]. However, following minor (subnephritogenic) mesangial injury, similar to that induced in the present study, the mesangial cells became susceptible to the mitogenic action of bFGF and PDGF *in vivo* and marked proliferation could be observed [51, 52]. This "priming" of the mesangial cells by subnephritogenic injury likely involved modulation of receptor expression and/or altered post-receptor responses [52, 54].

The above observations led us to test the effects of IL-6 in rats with prior minimal mesangial injury rather than in normal rats. As opposed to bFGF and PDGF, under these circumstances IL-6 had no detectable effect on glomerular cell activation (as assessed by the expression of α-smooth muscle actin and desmin [38]), cell proliferation or matrix accumulation. This was not due to biological inactivity of the infused IL-6 since it led to a considerable induction of the acute phase protein α_2-macroglobulin, similar to that observed in systemic inflammation induced by endotoxin injection [43]. However, we cannot exclude that higher, that is, pharmacological, doses of IL-6 might have yielded different biological responses under our experimental conditions.

IL-6 infusion in rats with anti-Thy 1.1 mesangioproliferative glomerulonephritis

In a fourth experimental approach, we tested whether or not the biological activity of IL-6 may depend on a synergism or interactions with other cytokines, such as IL-1, PDGF, bFGF or TGF-β, all of which are overexpressed in glomeruli of rats with anti-Thy 1.1 nephritis and/or have been invoked in its pathogenesis [51, 55–57]. Again, IL-6 had no significant effect on a large variety of damage parameters in rats with a fully established

mesangioproliferative glomerulonephritis. The only detectable effect of the IL-6 infusion, apart from augmenting the acute phase protein synthesis, was an up-regulation of glomerular matrix protein mRNA levels. These data corroborate previous *in vitro* findings in which IL-6 stimulation of mesangial cells also led to increased matrix protein transcription [14]. The lack of a detectable parallel increase of matrix protein deposition in our study may be due to either one of three possibilities: (a) relative insensitivity of our semiquantitative immunostaining scores to small increases in matrix protein deposition, (b) a post-transcriptional block in the matrix protein synthesis or effects of IL-6 on one of the various steps of matrix protein assembly, or (c) a concomitant increase in proteolytic activity. Independent of this latter issue, our data do not support a major role of IL-6 in mediating glomerular extracellular matrix accumulation *in vivo*. Similarly, in a murine model of mesangioproliferative IgA nephropathy, IL-6 administration had no adverse effects on the overall glomerular morphology or on the short-term course of the disease [58]. However, in this later study a roughly 35-fold lower total amount of IL-6 was administered (about 7.5 μg/kg body wt/5 days vs. 250 μg/kg/7 days in the present study). When given at the same dose as in the present study, IL-6 reduced proteinuria and macrophage activation in rats with nephrotoxic nephritis [59]. Part of this action may have resided in an IL-6 induced up-regulation of renal IL-1 type II receptor [59], which acts as a functional antagonist for the action of IL-1. In contrast, in murine models of lupus IL-6 has been identified as an important mediator of the nephritis [50, 60]. However, additional experiments demonstrated that this effect was largely mediated via IL-6 actions on the immune system rather than direct renal actions of the cytokine [50, 60].

In conclusion, using four different experimental approaches, we have failed to detect any evidence for a role of IL-6 in mediating mesangial cell activation or growth *in vivo*. Despite an induction of glomerular matrix protein transcription by IL-6 under special circumstances, we also failed to establish IL-6 as an important mediator of matrix protein accumulation. While each of the four different approaches individually cannot provide definitive evidence against a major role of IL-6 in modulating mesangial cell behavior *in vivo*, the consistency of our findings in four very diverse experimental situations strongly argues against an important role of IL-6 in the pathogenesis of mesangioproliferative diseases. This conclusion receives further support from recent observations in a murine model of IgA nephropathy [58]. Taken together, these experimental data at present do not support that neutralization or antagonism of IL-6 should be a therapeutical goal in mesangioproliferative glomerulonephritis.

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