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Expression of the chemokines MCP-1/CCL2 and RANTES/CCL5 is differentially regulated by infiltrating inflammatory cells

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Expression of the chemokines MCP-1/CCL2 and RANTES/CCL5 is differentially regulated by infiltrating inflammatory cells.

Background. Chemokines are involved in the regulation of the cellular renal infiltrate in glomerulonephritis; however, it is unclear to which degree resident glomerular cells or infiltrating leukocytes contribute to the formation of chemokines in glomerular inflammatory lesions. We therefore examined whether monocytes/macrophages play a role in the expression of the C-C chemokines MCP-1/CCL2 and RANTES/CCL5 in renal tissue in a lipopolysaccharide (LPS)-induced model of inflammation, where previously we have shown increased glomerular RANTES expression and glomerular infiltration of ED-1-positive cells.

Methods. Inflammatory lesions were induced by an intraperitoneal injection of LPS. The infiltration of monocytes into the glomerulus was reduced by two experimental approaches. First, rats were depleted of monocytes by the use of specific monocyte-antisera or by cytotoxic drugs. Second, the infiltration of monocytes into the kidney was reduced by using intercellular adhesion molecule-1 (ICAM-1) knockout mice.

Results. Both experimental approaches demonstrated a significant reduction in the number of infiltrating monocytes/macrophages after lipopolysaccharide injection. This reduction in the infiltration of inflammatory cells was associated with significantly reduced RANTES/CCL5 mRNA expression. However, MCP-1/CCL2 mRNA expression was not inhibited after the LPS injection by monocyte/macrophage depletion. Also, the increase in nuclear factor-κB (NF-κB) binding activity after the LPS injection was not reduced in pretreated animals. The experiments therefore demonstrate that infiltrating monocytes/macrophages contribute to increased RANTES/CCL5 mRNA expression in inflammatory renal lesions, whereas MCP-1/CCL2 mRNA expression and NF-κB activation were not reduced by monocyte/macrophage depletion.

Conclusion. MCP-1/CCL2 released from renal tissue upon stimulation plays a major role in the regulation of monocyte/macrophage infiltration, which contributes significantly to increased renal RANTES/CCL5 expression. This cross-talk between resident renal cells and monocytes/macrophages is therefore likely to boost the number of infiltrating inflammatory cells.

One of the hallmarks of early lesions in glomerular inflammation is infiltrating bone marrow-derived cells. Prominent cell types present in glomeruli are monocytes/macrophages. Several well-defined mechanisms contribute to the monocyte attachment and infiltration into the glomerulus [1–3], and recently much interest has focused on the role that chemokines might play [4, 5]. In different models of glomerular injury chemokines are expressed and secreted after the induction of the disease [6]. More convincingly, it has been demonstrated that if the biological effects of chemokines were inhibited by the use of chemokine antisera or chemokine receptor blockers, the influx of monocytes into the glomerulus is reduced [7–10].

The role of monocytes in the regulation of glomerular chemokine formation in vivo is currently unknown. This seems to be of major interest since inflammatory cell-derived chemokines could contribute to the amplification or potential resolution of the inflammatory process [11–13]. Increasing evidence shows that the interaction between monocytes and resident tissue cells or other inflammatory cells is important in the regulation of chemokine expression and secretion [14]. The mechanisms in this network have been partially elucidated in in vitro experiments and demonstrate, for example, that monocyte chemoattractant protein-1 (MCP-1)/CCL2 secretion is increased when monocytes are stimulated by two signals, one from the attachment to P-selectins and the other from stimulated platelets [15, 16].

The present experiments were designed to elucidate possible in vivo interactions between glomerular cells and infiltrating monocytes with respect to regulated upon activation, normal T cell expressed and secreted (RANTES)/CCL5 and MCP-1/CCL2 mRNA expression in a model of LPS-induced glomerular inflammation [17].

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Key words: glomerular inflammation, lipopolysaccharide, adhesion molecules, cell cross talk, monocyte chemoattractant peptide-1.

METHODS

Induction of renal inflammation

LPS model. Lipopolysaccharide (LPS, *Escherichia coli* type O111:B4; Sigma, Munich, Germany) was injected intraperitoneally (IP) in male Wistar rats (200 to 250 g body weight; Charles River Wiga, Germany). The dose used was 2 mg LPS/kg body weight.

Additional experiments were performed with intracellular adhesion moleule (ICAM-1) knockout or wild-type mice in which LPS was injected IP at a dose of 5 mg LPS/kg body weight. ICAM-$1^{-/-}$ mice (129Sv/J1 background backcrossed to Balb/c for seven generations) and ICAM-$1^{+/+}$ controls were maintained and bred in specific-pathogen-free (SPF) conditions, and were used for experiments at the age of 12 to 16 weeks. Their care was in accordance with institutional guidelines. The generation and characterization of the ICAM-1 knockout mice has been described in detail previously [18, 19].

For control experiments, phosphate-buffered saline (PBS) was injected IP and animals were left untreated or treated with normal rabbit IgG or an irrelevant antiserum (a-J) intravenously (IV; as a control for monocyte/macrophage antiserum) at the same time intervals.

Thy-1.1 nephritis model. Mesangial cell proliferative glomerulonephritis was induced by an IV injection of a rabbit anti-rat thymocyte antiserum. Technical aspects of the procedure and characteristics of this nephritis model have been described in detail previously [20, 21].

Monocyte/macrophage depletion

A monocyte/macrophage antiserum was prepared as described previously [22]. In brief, proteose peptone (10%)-stimulated peritoneal rat macrophages were harvested and rabbits immunized four times. The IgG fraction of the rabbit antiserum was prepared after heat inactivation of the serum (56°C, 1 h) by ammonium sulfate precipitation. The antiserum used was from a single pool and tested for efficiency by monocyte depletion in peripheral blood smears prior to its use in experiments. The antiserum was injected at minus 12 hours and at minus one hour before the LPS injection.

In additional experiments, a specific, adsorbed, rabbit anti-rat macrophage antiserum was used (Accurate Chemical, Westbury, NY, USA) [23]. The antiserum was injected at minus two days and at minus one hour before the LPS injection.

Additional rats were pretreated with nitrogen mustard (NM; 0.5 mg/kg body weight) once IV two days prior to LPS injection or cyclophosphamide (CyP) was administered IP at 70 mg/kg body weight on day minus 5 and at 35 mg/kg body weight at day minus 2 prior to the LPS application [24, 25].

In vitro incubation of renal tissue

Kidneys of non-treated ICAM-1 knockout and wild-type mice were harvested and further processed under a laminar flow for sterile conditions. Renal cortex was isolated by scissors and homogenized in a douncer. Cortical tissue single cell preparations were then cultured in RPMI supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin for 24 hours without stimulation or stimulated with 1 μg, 10 μg/mL LPS or with 10 μg LPS + 100 IU interferon-γ (IFN-γ)/mL medium. Thereafter, RNA was extracted for Northern blotting.

RNA isolation and Northern blot analysis

Glomeruli from freshly harvested rat kidneys were isolated by a sieving technique as described before [17]. In mice, cortex was isolated from kidneys and homogenized in a douncer.

Total RNA from isolated glomeruli of rats or from kidneys of mice was extracted by phenol-chloroform after direct lysis of glomeruli or kidney tissue in 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol. Twenty micrograms of total RNA was electrophoresed through a 1.2% agarose gel in running buffer [2.2 mol/L formaldehyde, 0.02 mol/L 3-(N-morpholino)-propanesulfonic acid, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA)]. The RNA was transferred onto nylon membranes (Hybond-N; Amersham Bioscience) and after UV-crosslinking prehybridized at 65°C for one hour in 0.1 × standard sodium citrate (SSC; 20 × SSC = 3 mol/L NaCl, 0.3 mol/L sodium citrate), 0.5% sodium dodecyl sulfate (SDS). The cDNA probe used were the following: MCP-1/CCL2 (a 577 bp Eco-R1 fragment), RANTES/CCL5 (an EcoRI-*XhoI* fragment of the murine RANTES clone pMuR3) and a 2.0 kb insert of pMCI encoding the murine 18 S RNA band as decribed previously [17, 20, 21]. The cDNA fragments were labeled with 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Bioscience) using a random priming kit (Oncor Appligene, Heidelberg, Germany). The membranes were hybridized with 10^6 cpm probe per mL in Rapid-Hyb buffer (Amersham Bioscience) for 24 hours at 70°C using a rotating drum. After hybridization blots were washed for 20 minutes in 2 × SSC (20 × SSC: 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0), and 0.1% SDS at room temperature, for at least 15 minutes in 0.4 × SSC in 0.1% SDS at 65°C and autoradiographed. Membranes were stripped with 5 mmol/L Tris-HCl, 0.2 mmol/L EDTA, 0.5% sodium pyrophosphate, and 5 × Denhardt's solution for 30 minutes at 65°C, and were subsequently rehybridized with the cDNA probe for the 18 S band to account for small loading and transfer variations. Exposed films were scanned with the Fluor-STM MultiImager and analyzed with the computer program 'Multianalyst' 1.1 or the hybridized membranes were

subjected to the GS-363 Molecular Imager System and 'Molecular Analyst' 1.5 (Bio-Rad Laboratories, Munich, Germany). Relative changes in RNA were calculated after assigning hybridization in control lanes a relative value of one. Samples were normalized for the signal intensity of the 18 S ribosomal RNA hybridization. Details of this procedure have been published previously [20, 21, 26].

Western blot analysis

Isolated glomeruli of kidneys were lysed in 100 μL Laemmli-buffer. Protein concentration was determined with the DC protein assay reagent (Bio-Rad Laboratories). One hundred micrograms of protein were loaded onto a 12.5% SDS-PAGE and semi-dry blotted onto a polyvinylidene difluoride (PVDF)-membrane (Hybond-P; Amersham Bioscience). The blots were blocked in 5% non-fat dry milk in PBS and 0.1% Tween-20 before incubating the blots for one hour in a 1:1000 dilution of the antibodies against ICAM-1 (R & D Systems, Wiesbaden, Germany) or PCNA (Transduction Laboratories, Heidelberg. Germany). After washes in PBS and 0.1% Tween-20, the blots were incubated for another hour in a 1:2000 dilution of rabbit anti-goat- or goat anti-mouse-horseradish peroxidase-linked IgG (Southern Biotechnology Associates, Birmingham, AL, USA) as the secondary antibody. The antibody-labeled proteins were detected with enhanced chemiluminescence (ECL) according to the manufacturer's description (Amersham Bioscience). Exposed films were scanned with the Fluor-STM MultiImager and analyzed with the computer program "Multianalyst" 1.1 (Bio-Rad Laboratories). Details of this technique have been published previously [20, 21].

Glomerular nuclear mini-extracts and EMSA

Kidneys were perfused with 150 mL sterile PBS per animal before harvesting. The nuclear mini-extracts were prepared and the electrophoretic mobility shift assay (EMSA) run according to the procedure described by Sakurai et al [27] with minor modifications. Briefly, isolated glomeruli from two animals were resuspended in 400 μL buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylenaglycol-bis (β-amino ether)-N,N′-tetraacetic acid (EGTA), 1 mmol/L dithiothreithol (DTT) and 0.5 mM phenylmethyl-sulfonylfluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin], homogenized with 50 strokes in a 7 mL glass-homogenizer (Wheaton, IL, USA) and chilled on ice for 15 minutes. After adding 25 μL of 10% Nonidet P-40 the homogenate was vigorously vortexed for 10 seconds and centrifuged at 15,000 × *g* for five minutes. To extract the nuclear proteins the nuclear pellet was resuspended in 100 μL buffer B [20 mmol/L HEPES (pH 7.9), 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT and 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin] and rocked at 400 rpm on ice on a shaking platform for 15 minutes. After centrifugation at 15,000 × *g* for five minutes the nuclear extracts were stored in aliquots at −80°C. Protein concentration was measured using the DC protein assay reagent (Bio-Rad Laboratories).

A nuclear factor-κB (NF-κB) consensus oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega, Heidelberg, Germany) was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Bioscience). One to 5 μg of nuclear protein was incubated for 30 minutes at room temperature with 100,000 cpm of the probe in 20 mmol/L HEPES (pH 7.9), 0.3 mmol/L EDTA, 0.2 mmol/L EGTA, 80 mmol/L NaCl and 2 μg Poly(dI-dC)Poly(dI-dC) (Amersham Bioscience) in a total volume of 20 μL. The DNA-protein complexes were subjected to electrophoresis on a 4% polyacrylamide gel that contains 2.5% glycerol, 6.7 mmol/L Tris-HCl (pH 7.5), 3.3 mmol/L sodium acetate and 0.1 mmol/L EDTA. After electrophoresis for 2.5 hours at 15°C the gel was vacuum-dried and autoradiographed.

Southwestern histochemistry

The Southwestern histochemistry was performed as described in detail previously [28]. NF-κB consensus oligonucleotides were digoxigenin labeled with a 3′-terminal transferase (Roche, Mannheim, Germany). Paraffin-embedded tissue sections were fixed in 0.5% paraformaldehyde and incubated with 0.1 mg/mL DNase I. The DNA-binding reaction was performed by incubation with 10 pmol of the labeled DNA probe in buffer containing 0.25% bovine serum albumin (BSA) and 0.5 μg/mL poly(dI-dC). The sections were then incubated with alkaline phosphatase-conjugated anti-dioxigenin antibody and colorimetric detection was performed. Preparations without probe were used as negative controls, and mutant-labeled probe and excess of unlabeled probe were used to test the specificity of the technique.

Morphology

Kidney samples were fixed in 4% buffered formalin and embedded in paraffin. For light microscopy 4-μm thick sections were stained with periodic acid-Schiff (PAS). Immunohistochemistry was performed with ED-1 antibody (Chemicon, CA, USA) for rats and F4/80 or MCA519 antibodies (Serotec, München, Germany) for mice to determine kidney infiltrating monocytes/macrophages. Alkaline phosphatase-antialkaline phosphatase (APPAP; Camon, Wiesbaden, Germany) was used as the detection system. Technical details of the morphological procedures have been outlined previously [20, 26]. To count the number of infiltrating monocytes/macrophages per glomerular cross sectional area, at least 30 glomeruli were evaluated from each kidney in a blinded fashion. The amount of monocyte/macrophage infiltration into

the tubulointerstitium of the kidney was determined by counting the number of positively stained cells per 20 visual fields (magnification ×720).

Experimental design

Rat experiments. The following groups of animals were examined:

(1) *Untreated.* LPS or for controls PBS was injected intraperitoneally.
(2) *Pretreated with cytotoxic drugs.* Additional animals were pretreated two days prior to LPS application with nitrogen mustard intravenously or five and two days prior to LPS injection with cyclophosphamide intraperitoneally.
(3) *Pretreated with antisera.* Another group of animals was pretreated with rabbit anti rat monocyte/macrophage antisera intravenously before LPS injection. Controls were treated with IV injected normal rabbit serum.

From controls and at 1, 3 and 6 hours after LPS injection, the kidneys were harvested and further processed for morphological examinations and glomerular isolation of RNA, for glomerular cytoplasmic protein extraction or for glomerular preparation of nuclear proteins.

Two rats were used for morphology, for glomerular RNA extraction three rats, glomerular cytoplasmic protein extraction two rats, and for glomerular nuclear protein preparation two rats were examined. The experiments were repeated two to three times.

Mouse experiments. The following groups of animals were examined:

(1) *In vivo experiments.* LPS or for controls PBS was injected intraperitoneally in either ICAM-1 wild-type or knockout mice. For morphology kidneys of two mice were examined from controls and 3, 6, 12 and 24 hours after an IP LPS-injection in wild-type and knockout animals. At 12 and 24 hours after the LPS application kidneys were harvested from additional ICAM-1 wild-type and knockout mice ($N = 3$) and the cortex homogenized for RNA extraction. A pool of kidneys taken from 2 to 3 animals was examined and experiments were repeated three times.
(2) *Ex vivo experiments.* Additional in vitro experiments were performed. For this purpose kidneys ($N = 2$ animals) were harvested under sterile conditions and single cell suspensions prepared from cortex both from ICAM-1 wild-type and knockout animals. Single cell suspensions were incubated in vitro with or without further stimulation with LPS or LPS and IFN-γ for 24 hours before RNA extraction. The experiments were repeated twice.

Table 1. Efficiency of monocyte/macrophage depletion in rat experiments

	Peripheral blood monocytes/nl	ED1-positive cells/ glomerular cross sectional area
Control	490 ± 120	0.2 ± 2.1
LPS	610 ± 175	4.5 ± 1.8[a]
LPS + a-M/M	80 ± 45[c]	1.0 ± 1.5[b]
LPS + NM	35 ± 22[c]	0.3 ± 1.8[c]
LPS + CyP	22 ± 15[c]	ND
LPS + a-RM	18 ± 11[c]	ND

To demonstrate for efficiency of the pretreatment protocols used in rats to deplete monocytes/macrophages peripheral blood monocytes and ED-1 positive cells per glomerular cross-sectional area were counted in controls and 6 hours after intraperitoneal LPS-injection ($N = 3$). Animals were pretreated with rabbit anti-rat monocyte/macrophage antisera (a-M/M or a-RM) or with cytotoxic drugs (NM or CyP). Abbreviations are in the Appendix; ND is not done.

[a] $P < 0.05$ vs. control

[b] $P < 0.05$ and [c] $P < 0.01$ when compared with LPS-injected otherwise non-pretreated rats

Statistical analysis

Results are expressed as means ± SEM. For statistical comparison the Mann-Whitney U test was performed. For multiple group comparisons, the non-parametric Kruskal-Wallis test was used. P values <0.05 were considered as statistically significant. For Northern blot analysis an increase or decrease of RNA expression as determined by densitometry of at least 25% was considered significant ($P < 0.05$) and analyzed further with the Kruskal-Wallis test.

RESULTS

Monocyte/macrophage depletion

To test for efficiency of monocyte/macrophage depletion using rabbit anti-rat monocyte/macrophage antisera or cytotoxic drugs differential peripheral blood cell counts for monocytes were performed in control rats and rats six hours after the injection of LPS (Table 1). In the same animals the number of ED-1-positive cells per glomerular cross sectional area also was determined (Table 1). To exclude non-specific side effects of either the monocyte/macrophage antiserum or of the cytotoxic drug, two different monocyte/macrophage antisera were used, one produced in our in-house animal facilities (monocyte/macrophage antiserum) and one commercially available (a-RM); two different cytotocix drugs (nitrogen mustard and cyclophosphamide) also were used.

As shown in Table 1, the number of circulating peripheral monocytes was significantly reduced ($P < 0.01$) six hours after LPS injection in animals pretreated with either the antisera or the cytotoxic drugs. Also, the number of monocytes/macrophages infiltrating the glomerulus was significantly reduced (P at least <0.05) in pretreated animals at six hours after LPS injection. Our prior study demonstrated the maximum of inflammatory cell infiltration into the glomerulus after LPS-application at this time point [17].

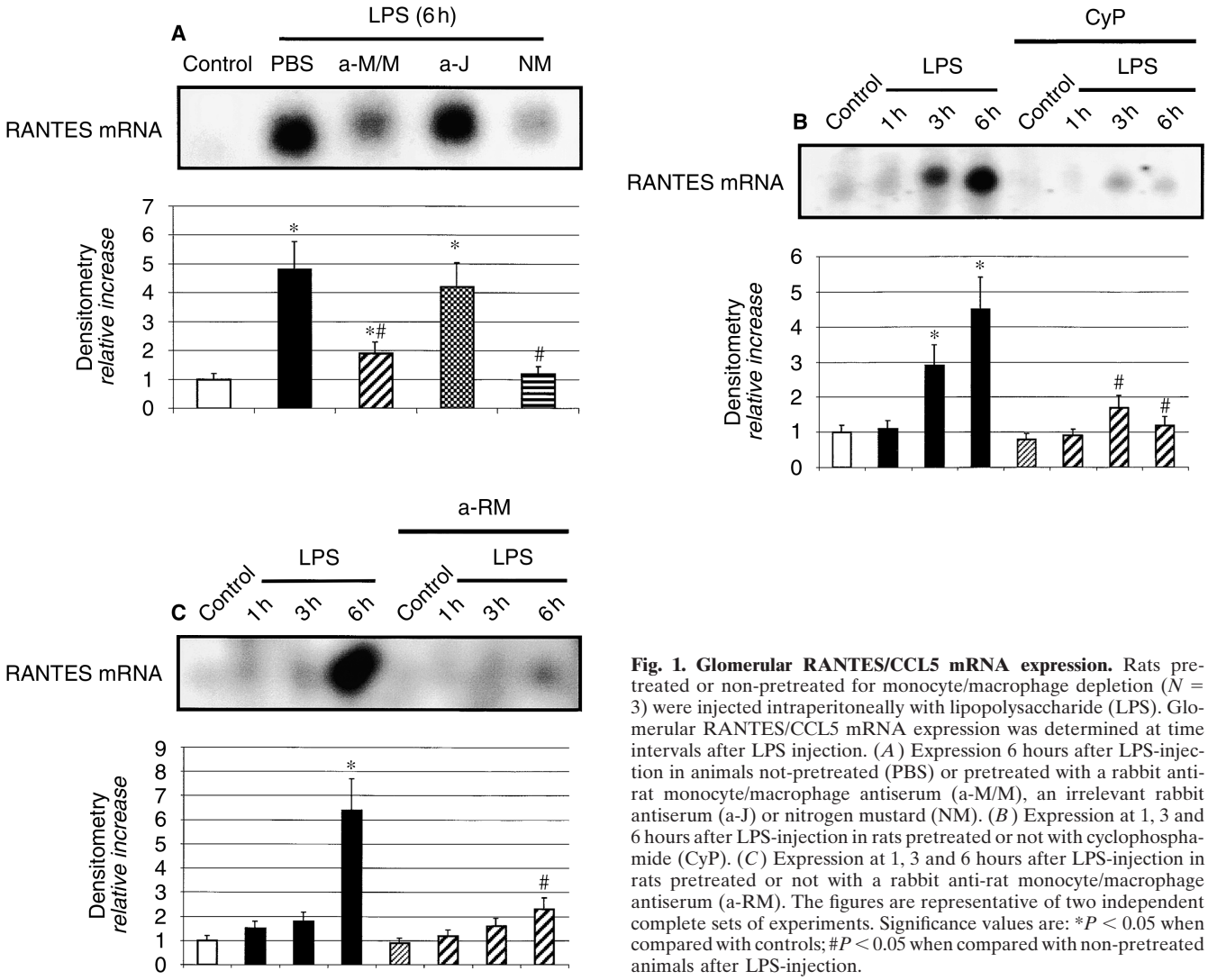


Fig. 1. Glomerular RANTES/CCL5 mRNA expression. Rats pretreated or non-pretreated for monocyte/macrophage depletion ($N = 3$) were injected intraperitoneally with lipopolysaccharide (LPS). Glomerular RANTES/CCL5 mRNA expression was determined at time intervals after LPS injection. (*A*) Expression 6 hours after LPS-injection in animals not-pretreated (PBS) or pretreated with a rabbit anti-rat monocyte/macrophage antiserum (a-M/M), an irrelevant rabbit antiserum (a-J) or nitrogen mustard (NM). (*B*) Expression at 1, 3 and 6 hours after LPS-injection in rats pretreated or not with cyclophosphamide (CyP). (*C*) Expression at 1, 3 and 6 hours after LPS-injection in rats pretreated or not with a rabbit anti-rat monocyte/macrophage antiserum (a-RM). The figures are representative of two independent complete sets of experiments. Significance values are: $*P < 0.05$ when compared with controls; $\#P < 0.05$ when compared with non-pretreated animals after LPS-injection.

Glomerular RANTES/CCL5 mRNA expression

Glomerular RANTES/CCL5 mRNA expression increased rather late after LPS injection, with a maximum at six hours when compared with control rats (Fig. 1 A, B).

When animals were pretreated to deplete monocytes/macrophages before LPS injection, the glomerular RANTES/CCL5 mRNA expression was significantly reduced when compared with non-pretreated rats after LPS injection. This was shown by the use of cytotoxic drugs and monocytes/macrophage antisera. Nitrogen mustard (Fig. 1A) and cyclophosphamide (Fig. 1B) significantly reduced glomerular RANTES/CCL5 mRNA expression at three and six hours after LPS-injection. Also, the rabbit anti-rat monocyte/macrophage antisera used significantly reduced glomerular RANTES/CCL5 mRNA expression (Fig. 1 A, C). This effect was specific for monocyte/macrophage depleting antisera, as an irrelevant antiserum (a-J) did not significantly influence glomerular RANTES/CCL5 expression (Fig. 1A).

Glomerular MCP-1/CCL2 mRNA expression

MCP-1/CCL2 mRNA expression was already increased at one hour after LPS injection and was consistently elevated throughout the six-hour observation period when compared with controls (Fig. 2 A, B).

Pretreating animals for monocyte/macrophage depletion with either cyclophosphamide (Fig. 2A) or with a specific antiserum (Fig. 2B) did not reduce glomerular MCP-1/CCL2 mRNA expression. We found a slight, albeit not consistent increase of glomerular MCP-1/CCL2 expression at one hour after LPS-injection in cyclophosphamide pretreated rats when compared with non-pretreated animals (Fig. 2A). At three and six hours after LPS-application, the MCP-1/CCL2 expression was not different in cyclophosphamide-pretreated animals when compared with non-pretreated rats (Fig. 2A). Also, a rabbit anti-rat monocyte/macrophage antiserum did not reduce glomerular MCP-1/CCL2 mRNA expression at three and six hours after LPS-injection (Fig. 2B).

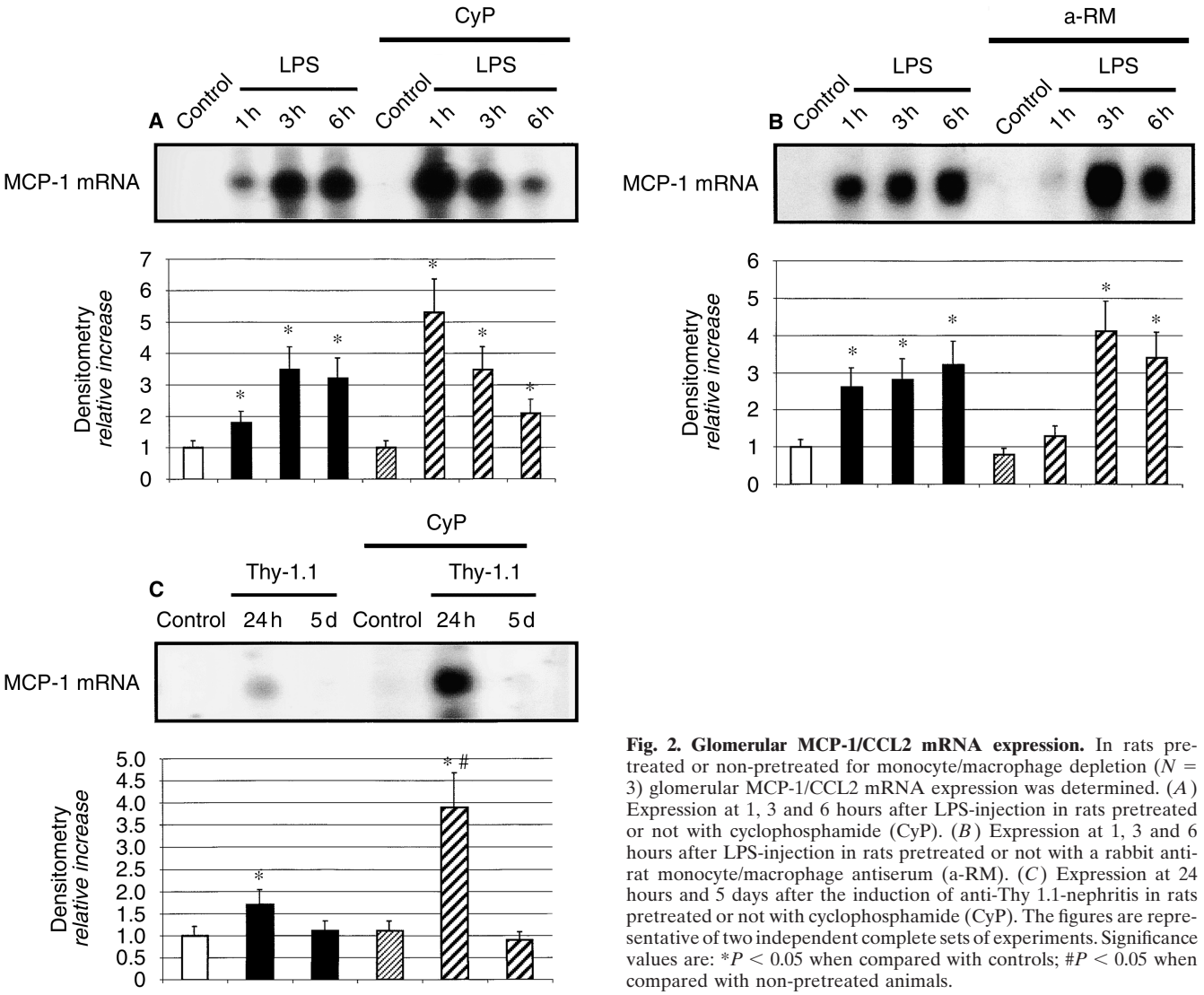


Fig. 2. Glomerular MCP-1/CCL2 mRNA expression. In rats pretreated or non-pretreated for monocyte/macrophage depletion ($N = 3$) glomerular MCP-1/CCL2 mRNA expression was determined. (*A*) Expression at 1, 3 and 6 hours after LPS-injection in rats pretreated or not with cyclophosphamide (CyP). (*B*) Expression at 1, 3 and 6 hours after LPS-injection in rats pretreated or not with a rabbit anti-rat monocyte/macrophage antiserum (a-RM). (*C*) Expression at 24 hours and 5 days after the induction of anti-Thy 1.1-nephritis in rats pretreated or not with cyclophosphamide (CyP). The figures are representative of two independent complete sets of experiments. Significance values are: $*P < 0.05$ when compared with controls; $\#P < 0.05$ when compared with non-pretreated animals.

In order to test whether these findings are generalizable for other inducers of glomerular injury that are more specifically targeted for the kidney, the Thy-1.1 nephritis model was used. There was a significant increase in glomerular MCP-1/CCL2 mRNA expression at 24 hours after the IV injection of the anti-Thy-1.1 antibody, as also described previously (Fig. 2C) [20, 21]. Depleting animals of monocytes/macrophages by cyclophosphamide administration before the induction of the Thy-1.1 nephritis did not reduce, but rather significantly increased glomerular MCP-1/CCL2 expression at 24 hours when compared with non-pretreated nephritic rats (Fig. 2C).

Glomerular ICAM-1 and PCNA expression

To determine whether the interventional treatment of the animals for monocyte/macrophage depletion also changed the expression of the adhesion molecule ICAM-1 or the proliferation marker PCNA, glomerular protein expression was determined at six hours after LPS injection in pretreated and non-pretreated rats.

Glomerular ICAM-1 protein expression is significantly increased at six hours after LPS injection when compared with control rats ($P < 0.01$). This increase was not reduced in animals pretreated with the rabbit anti-rat monocyte/macrophage antiserum or nitrogen mustard (NM) (Fig. 3).

As a marker of glomerular proliferation PCNA protein expression was determined by Western blotting. PCNA protein was significantly increased at six hours after LPS injection when compared with control rats ($P < 0.01$). This LPS-induced increase in glomerular proliferation was not changed in monocyte-depleted animals using a rabbit anti-rat monocyte/macrophage antiserum (Fig. 3). With NM the glomerular PCNA protein expression was, however, significantly reduced at six hours

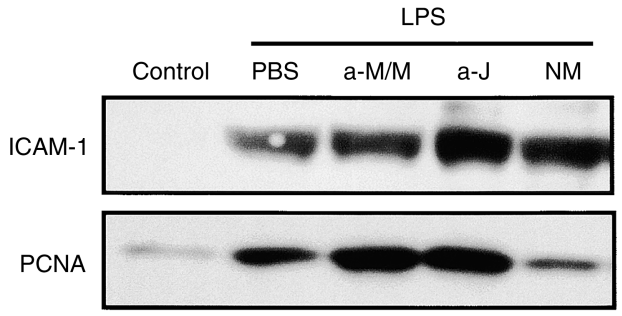


Fig. 3. Glomerular intracellular adhesion molecule-1 (ICAM-1) and proliferating cell nuclear antigen (PCNA) protein expression. In rats pretreated or non-pretreated for monocyte/macrophage depletion ($N = 2$) glomerular protein expression was determined by Western blotting. Expression was determined at 6 hours after LPS injection in animals not-pretreated (PBS) or pretreated with a rabbit anti-rat monocyte/macrophage antiserum (a-M/M), an irrelevant rabbit antiserum (a-J) or nitrogen mustard (NM). The figure is representative of three independent complete sets of experiments.

after LPS injection when compared with non-pretreated animals ($P < 0.05$). This indicates that NM not only reduced the proliferation of bone marrow derived cells, but also reduced the proliferation of glomerular cells.

Effect of monocyte/macrophage depletion on glomerular NF-κB activation

The activation of transcription factors plays a pivotal role for the regulation of chemokine expression. As shown by others in in vitro and in vivo studies, the activation of the transcription factor nuclear factor-κB (NF-κB) plays a major role in the regulation of MCP-1/CCL2 and RANTES/CCL5 expression [29]. Therefore, to further elucidate possible mechanisms by which glomerular RANTES mRNA expression might be reduced by monocyte/macrophage depletion, the activation of the transcription factor NF-κB was examined.

Glomerular NF-κB DNA binding activity significantly increased at one, three and six hours after LPS-injection when compared with controls with a maximum at three hours (Fig. 4). Pretreating rats for monocyte/macrophage depletion with NM (Fig. 4A; lane 3 vs. lane 2) or cyclophosphamide (CyP; Fig. 4B) did not significantly reduce glomerular NF-κB DNA binding activity. With the use of rabbit anti-rat monocyte/macrophage antisera there was a trend, albeit not significant, to an even enhanced glomerular NF-κB DNA binding activity at all time points examined after LPS injection (Fig. 4C).

In addition to gel shift experiments, the activation of the transcription factor NF-κB was analyzed in situ by Southwestern histochemistry in paraffin embedded tissue sections to further evaluate which glomerular cell population, resident cells or infiltrating inflammatory cells contribute to increased NF-κB binding activity after LPS injection. As shown in Figure 5, there was a significant increase in glomerular NF-κB expression three hours after LPS injection when compared with controls. By double staining with the monocyte-specific antiserum ED-1, NF-κB activation could be detected both in resident glomerular cells and infiltrating monocytes/macrophages (Fig. 5).

Experiments in ICAM-1 knockout mice

To further examine the role that inflammatory infiltrating cells might play in renal RANTES/CCL5 mRNA expression, ICAM-1 knockout mice were used. It has been shown previously that the number of infiltrating inflammatory cells is significantly reduced in ICAM-1 knockout animals after the induction of inflammatory lesions [18, 19, 30].

We first reproduced these findings in our model of LPS-induced renal inflammation by morphological examinations. Monocytes/macrophages stained positive with F4/80 or MCA519 antibodies (Serotec, Wiesbaden, Germany) in glomeruli and tubulointerstitium after LPS injection, as shown at six hours (Fig. 6A). Counting cells at 3, 6, 12 and 24 hours after LPS injection demonstrated a significant reduction of the number of infiltrating monocytes/macrophages in glomeruli and interstitum in ICAM-1 knockout mice when compared with wild-type animals (Fig. 6B).

The mRNA expression of renal RANTES/CCL5 then was determined in mice. As shown for the experiments in rats, reducing the number of infiltrating mononuclear cells into the kidney was associated with a reduced RANTES/CCL5 mRNA expression. A significant reduction of RANTES/CCL5 expression in ICAM-1 knockout mice was found when compared with wild-type animals at 12 and 24 hours after LPS injection, as shown in Figure 7.

To exclude the possibility that renal tissue from ICAM-1 knockout animals is less responsive to LPS than renal parenchymal cells taken from ICAM-1 wild-type animals, kidneys from both knockout and wild type animals were isolated, single cell suspensions prepared, and these cells incubated in vitro with LPS and LPS + IFN-γ. The experiments demonstrated a significant increase in RANTES/CCL5 mRNA expression in renal parenchymal cells taken from ICAM-1 wild-type and knockout animals when compared with cell populations before LPS was added to the medium. There was no significant difference in RANTES/CCL5 expression when single cell suspension taken from wild-type animals was compared with knockout mice (Fig. 8). These results are in accordance with other studies demonstrating that circulating mononuclear cells of ICAM-1 knockout mice respond similarly to cells taken from ICAM-1 wild-type animals to an inflammatory stimulus [31, 32].

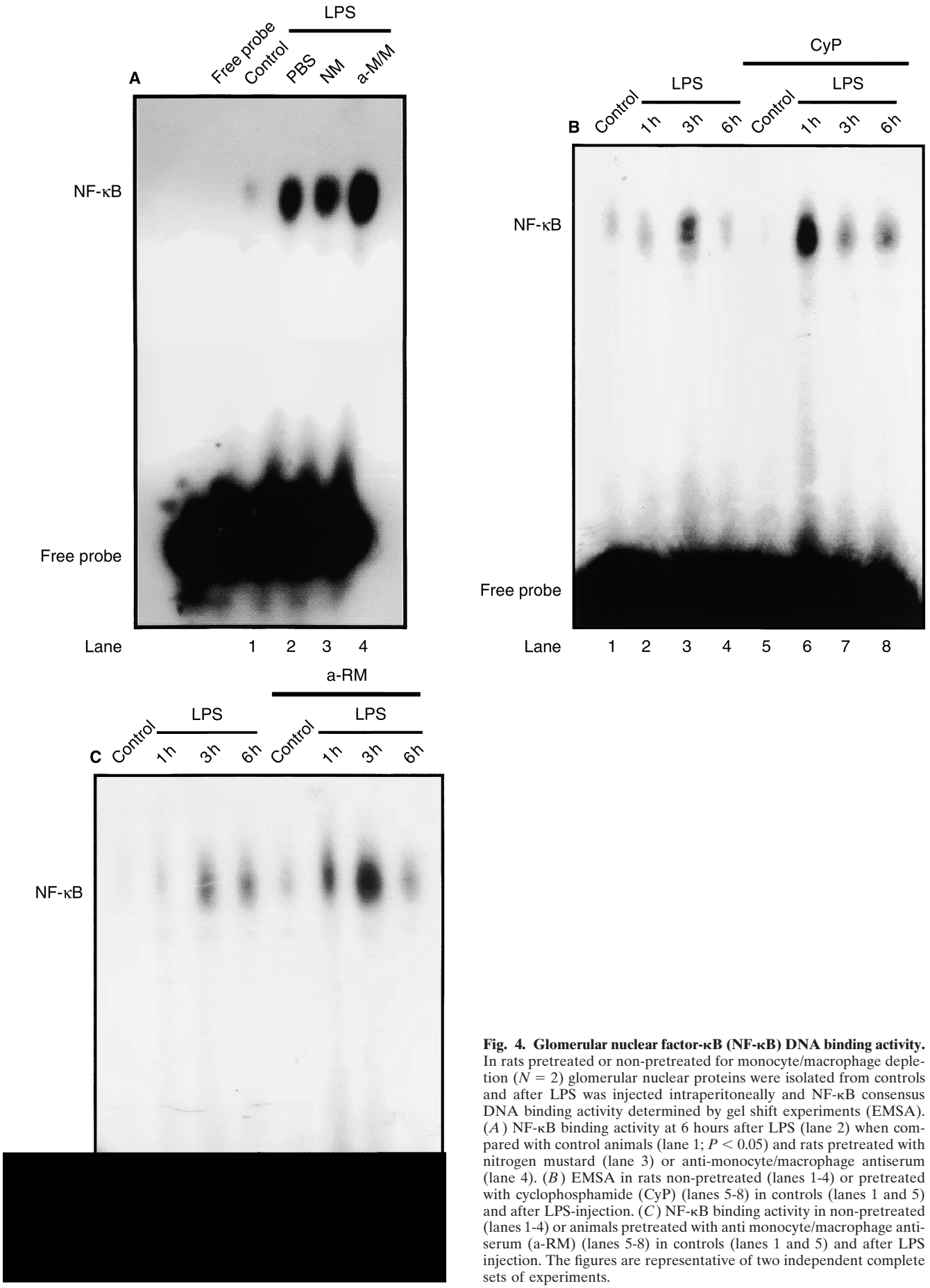


Fig. 4. Glomerular nuclear factor-κB (NF-κB) DNA binding activity. In rats pretreated or non-pretreated for monocyte/macrophage depletion ($N = 2$) glomerular nuclear proteins were isolated from controls and after LPS was injected intraperitoneally and NF-κB consensus DNA binding activity determined by gel shift experiments (EMSA). (*A*) NF-κB binding activity at 6 hours after LPS (lane 2) when compared with control animals (lane 1; $P < 0.05$) and rats pretreated with nitrogen mustard (lane 3) or anti-monocyte/macrophage antiserum (lane 4). (*B*) EMSA in rats non-pretreated (lanes 1-4) or pretreated with cyclophosphamide (CyP) (lanes 5-8) in controls (lanes 1 and 5) and after LPS-injection. (*C*) NF-κB binding activity in non-pretreated (lanes 1-4) or animals pretreated with anti monocyte/macrophage antiserum (a-RM) (lanes 5-8) in controls (lanes 1 and 5) and after LPS injection. The figures are representative of two independent complete sets of experiments.

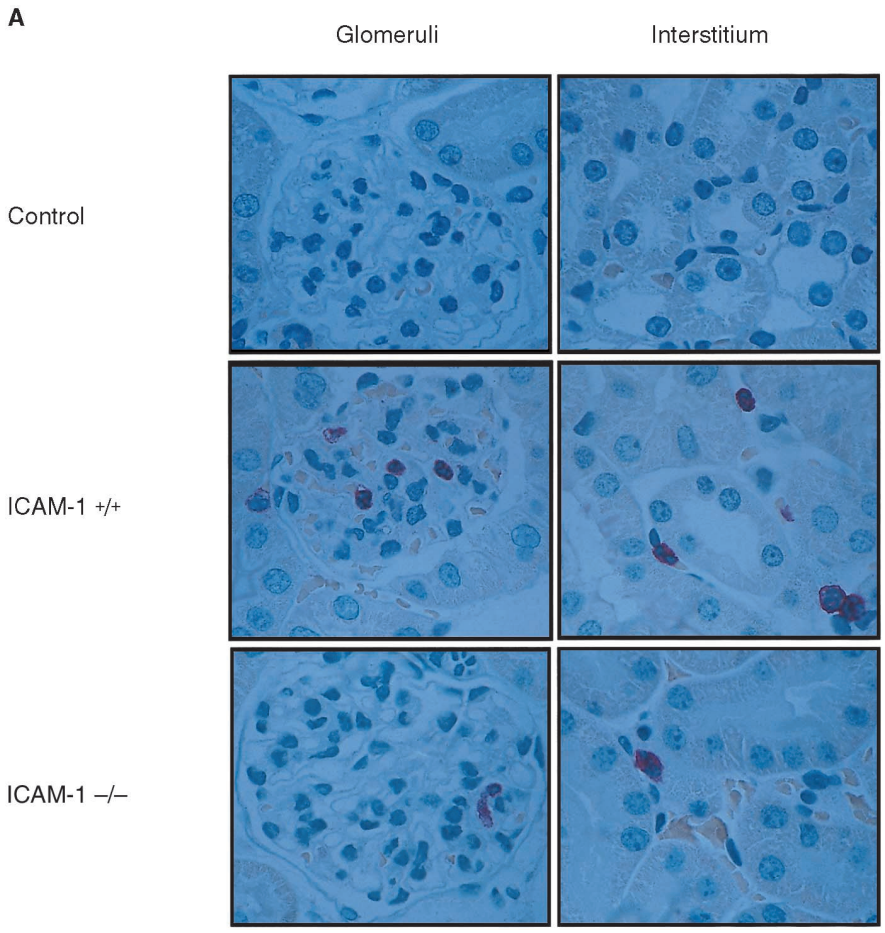
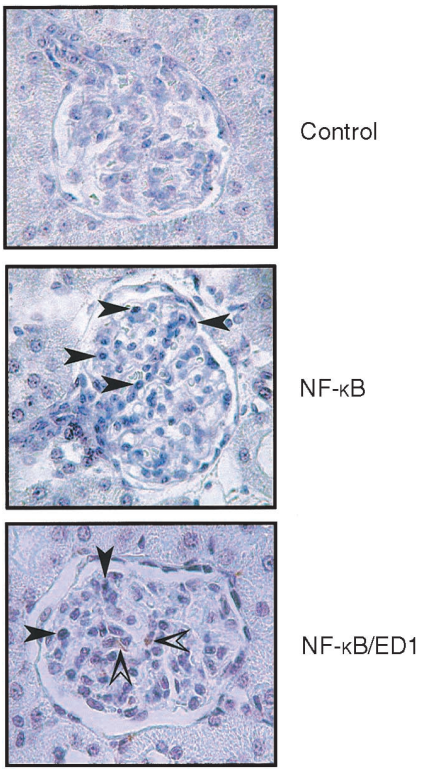


Fig. 6. Staining for monocytes/macrophages in LPS-treated mice. Renal tissue from ICAM-1 wild type and ICAM-1 knockout mice ($N = 2$) was stained for MCA519-positive monocytes/macrophages in controls and 3, 6, 12 and 24 hours after LPS injection. (*A*) Representative sections are given for glomeruli and tubulointerstitium at 6 hours after LPS injection. (Reproduction of this figure in color was made possible by a grant from Aventis Pharma, Bad Soden, Germany.)



DISCUSSION

Chemokines play a pivotal role in directing migration and adhesion of infiltrating cells to an inflammatory lesion. Inhibition of chemokine expression or secretion significantly reduced cell infiltration and injury. These mechanisms also apply for renal diseases. Abolishing the effect of chemokines by the use of specific antisera reduced the number of cells infiltrating the glomerulus in different models of glomerulonephritis [6, 9, 10, 33].

Chemokines are expressed and secreted upon stimulation by resident tissue cells such as mesangial cells, and also by inflammatory cells such as monocytes/macrophages. The release of chemokines from these cells also is regulated by costimulatory mediators [5, 6, 11]. For

Fig. 5. Southwestern histochemistry for NF-κB. In controls and rats 3 hours after LPS injection ($N = 2$) NF-κB binding was localized in the glomeruli by Southwestern histochemistry. In additional experiments double staining for ED1-positive monocytes/macrophages was performed. Closed arrows indicate nuclear localization of NF-κB, and open arrows indicate cells that stained positive for NF-κB and simultaneously ED-1 (magnification ×720). (Reproduction of this figure in color was made possible by a grant from Aventis Pharma, Bad Soden, Germany.)

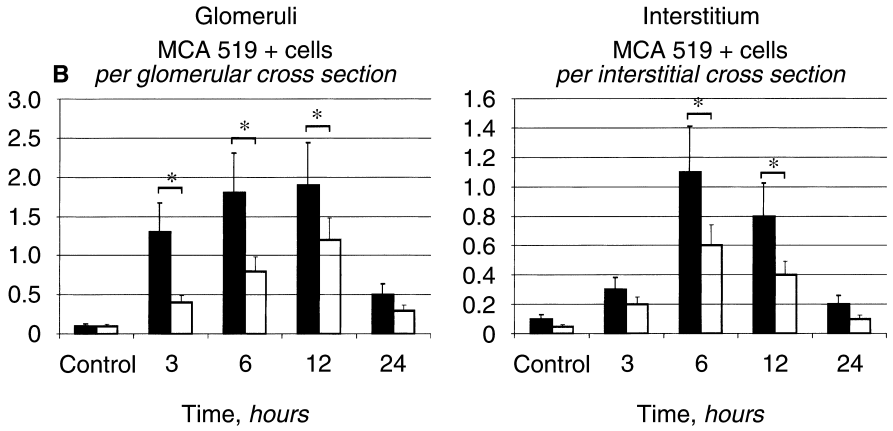


Fig. 6. (Continued) (*B*) Quantification of MCA519-positive cells per glomerular cross sectional and interstitial areas in time intervals after LPS-injection. Symbols are: (■) ICAM-1 wild-type mice; (□) ICAM-1 knockout mice; $*P < 0.05$ when wild-type animals were compared with ICAM-1 knockout mice.

example, MCP-1/CCL2 released from monocytes adherent to the adhesion molecule P-selectin is further increased by mediators released from activated platelets [15, 16]. Cross-linking of ICAM-1 also induced interleukin-8 (IL-8)/CXCL8 and RANTES/CCL5 expression in human endothelial cells [34]. Thus, the amount of chemokines released at an inflammatory site depends on a cross talk between infiltrating inflammatory and resident tissue cells. This cross talk is likely to substantially influence the outcome of the lesion.

The present experiments thus focused on infiltrating monocytes to investigate their potential role with respect to glomerular MCP-1/CCL2 and RANTES/CCL5 mRNA expression. Our results demonstrate that glomerular RANTES/CCL5 mRNA expression depends on monocytes infiltrating the glomerulus. Depletion of monocytes before the induction of the inflammatory lesion by LPS significantly reduced glomerular RANTES/CCL5 expression (Fig. 1). This was seen with specific rat monocyte/macrophage antisera and with cytotoxic drugs (nitrogen mustard or cyclophosphamide). Glomerular inflammatory responses to LPS were, however, not unspecifically reduced by the treatment protocols. First, glomerular MCP-1/CCL2 mRNA expression significantly increased at one hour after LPS injection and remained significantly elevated throughout the six-hour observation period (Fig. 2). This increased glomerular MCP-1/CCL2 mRNA expression was not reduced by the treatment protocols using either monocyte/macrophage antisera or cytotoxic drugs for cell depletion. This is in accordance with previously published data in the zymosan peritonitis model in mice demonstrating that macrophage depletion did not modify the amounts of MCP-1 released in the lavage fluid [35].

Second, glomerular ICAM-1 expression was significantly increased six hours after LPS application and neither the monocyte/macrophage antiserum or NM reduced this increased expression, and also an increased proliferative response after LPS injection as determined

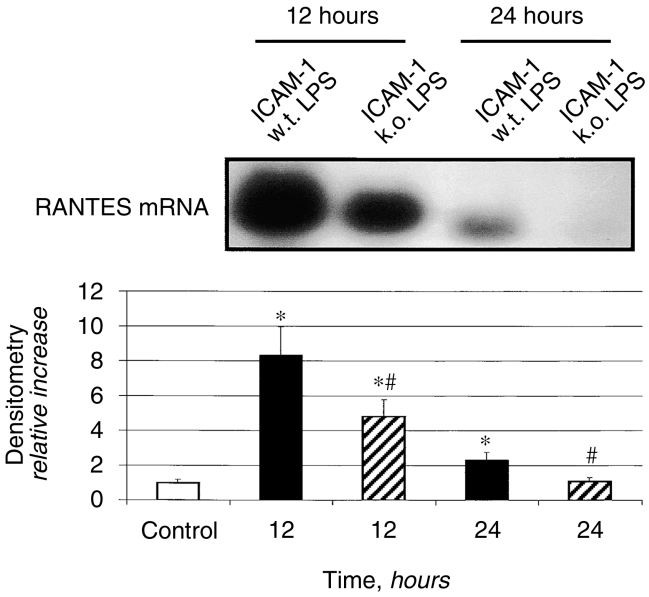


Fig. 7. Renal RANTES/CCL5 mRNA expression in ICAM-1 knockout and wild-type mice. RNA was extracted from renal tissue of mice ($N = 3$) at 12 and 24 hours after LPS was injected intraperitoneally. This figure is representative of three independent complete sets of experiments. Significance values are: $*P$ at least <0.05 when compared with controls; $^{\#}P < 0.05$ when compared with wild-type animals after LPS-injection.

by PCNA protein expression was not reduced by the antiserum (Fig. 3). Nitrogen mustard reduced glomerular PCNA expression, however, this might be due to nonspecific side effects of NM as it not only inhibits proliferation of bone marrow derived cells, but also might induce growth arrest of glomerular cells.

In an anti-GBM model of glomerulonephritis, Lefkowith and coworkers have previously demonstrated that the glomerular expression of CC and CXC chemokines is dependent on the presence of polymorphonuclear leukocytes (PMN) and monocyte/macrophage. In the early phase of this model chemokine expression was PMN dependent, and in the later or sustained phase ex-

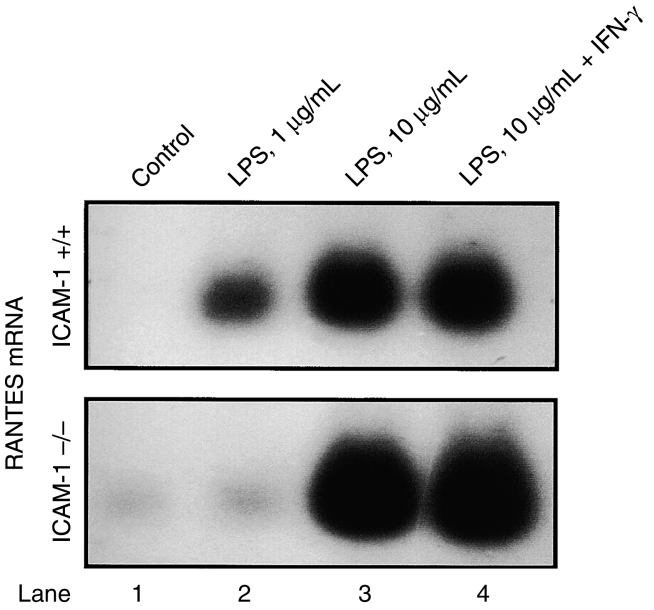


Fig. 8. RANTES/CCL5 mRNA expression in renal parenchymal cells after in vitro LPS-stimulation. Renal parenchymal single cell suspensions were prepared from ICAM-1 knockout and wild-type mice and stimulated in vitro for 24 hours with LPS or a combination of LPS and IFN-γ. When compared with controls (lane 1) there was a significant increase ($P < 0.01$) in RANTES/CCL5 mRNA expression after stimulation with high doses of LPS or the combination of LPS and IFN-γ (lanes 3 and 4). The increase in RANTES/CCL5 expression at a low LPS concentration in wild-type animals (lane 2) was not consistently reproduced and might be due to some contamination during the single cell preparation procedure. The figure is representative of two independent complete sets of experiments.

pression of chemokines was monocyte/macrophage dependent [36]. These data together with the present findings demonstrate that glomerular chemokine expression involves a complex network of factors between resident and infiltrating inflammatory cells. This positive feedback loop between glomerular cells and infiltrating inflammatory cells further increases chemokine gene expression and hence might boost the number of infiltrating inflammatory cells. These in vivo data are supported by recently published in vitro data where in co-culture experiments the interaction between monocytes and fibroblasts or monocytes and astrocytes were shown to increase MCP-1/CCL2 production [37, 38]. Physically separating the two cell types by a semipermeable membrane precluded MCP-1/CCL2 production. Thus, the interaction of resident cells with infiltrating inflammatory cells by adhesion molecules and their ligands may coamplify chemokine production. It also has been demonstrated recently that RANTES/CCL5 released from platelets after stimulation binds to activated endothelial cells and enhances the firm adhesion of monocytes to the endothelium [39]. These data together with the present findings support the pathophysiological concept that inflammatory cells further boost the inflammatory response in the tissue by increasing the local amount of chemokines released.

Further support of this pathogenetic link comes from the present experiments performed in ICAM-1 knockout mice. In these animals the LPS-induced infiltration of monocytes into the kidney was significantly reduced at all time points examined (Fig. 6). The reduced number of monocytes infiltrating the kidney parallels the significantly reduced RANTES/CCL5 gene expression in ICAM-1 knockout animals when compared with wild-type mice (Fig. 7). However, when incubating single cells taken from kidney cortex homogenates in vitro with LPS or a combination of LPS and IFN-γ for 24 hours, RANTES/CCL5 gene expression was significantly increased when compared with non-treated controls and there were no significant differences between wild-type and knockout mice (Fig. 8). These data demonstrate that resident renal cells respond with a similar increase in RANTES gene expression after in vitro stimulation. Thus, reduced RANTES gene expression found in ICAM-1 knockout mice in vivo strictly correlates with the reduced number of infiltrating monocytes.

Adhesion molecules and their ligand counterparts play an important role in the recruitment of cells to inflammatory lesions, in signal transduction and regulation of chemokine expression [40, 41]. ICAM-1 knockout animals have been an exciting tool to investigate mechanisms of inflammatory processes in in vivo experiments [19, 30, 42–44]. A reduced inflammatory response and tissue injury in the kidney has been described in ICAM-1 knockout animals, for example, when heterologous and autologous models of anti-GBM disease were examined. In these models improved glomerular morphology after the induction of the disease strictly correlated with a reduced number of infiltrating inflammatory cells. Also, in a model of systemic autoimmune disease in the MRL/MpJ-Fas^{lpr} mice, additional ICAM-1 deficiency resulted in a significant reduction in vasculitis in the kidney through impaired neutrophil and/or T cell adhesion [45]. Treatment with a monoclonal antibody to ICAM-1 also reduced proteinuria in a rat model of in situ immune complex glomerulonephritis [46]. Recent experiments also demonstrate that cell-to-cell contact between T cells and renal tubular epithelial cells mediated in part by LFA-1/ICAM-1 interactions are involved in RANTES/CCL5 production, a pathogenic role of which has been proposed for interstitial mononuclear cell infiltration previously [14, 47].

Chemokines are not stored at the cellular level. The regulation of chemokine expression is mostly due to a new and rapid transcription, which is tightly regulated upon binding of transcription factors to the promoter regions. Therefore, to elucidate possible mechanisms by which infiltrating glomerular cells might reduce glomerular RANTES/CCL5 gene expression, we examined the

activation of the transcription factor NF-κB in isolated glomeruli taken from rats one, three and six hours after LPS-injection. RANTES/CCL5 and MCP-1/CCL2 mRNA expression are regulated by NF-κB as shown previously by others [48, 49]. Systemic LPS induced NF-κB activation in ex vivo isolated glomerular nuclear proteins (Fig. 4). This NF-κB activation was not reduced in animals pretreated with a specific anti-monocyte antiserum or cytotoxic drugs (cylclophosphamide). Thus, reducing the number of infiltrating monocytes/macrophages did not significantly change NF-κB activation in glomeruli after LPS injection. This finding is supported by recently published in vitro data. Co-incubating mesangial cells and macrophages induced NF-κB activation preferentially in mesangial cells [50]. Activation of NF-κB has been demonstrated previously in experimental inflammatory renal diseases and in vitro when mesangial and tubular cells were stimulated with inflammatory cytokines. Mechanisms of NF-κB activation in these models have been elucidated partially [51–54]. Our in vivo data now demonstrate, that LPS is a strong inducer of NF-κB activation in glomeruli. Reduced RANTES/CCL5 mRNA expression in this model therefore is not due to a lack of NF-κB activation, although it has been shown recently that the regulation of RANTES/CCL5 expression is more complex and involves both a stimulus and tissue specific coordinate activation of different transcription factors [55].

Our data demonstrate that infiltrating inflammatory cells contribute to renal RANTES/CCL5 mRNA expression whereas MCP-1/CCL2 is mainly released from renal parenchymal cells upon appropriate stimulation and its expression is not influenced by infiltrating inflammatory cells. This positive feedback loop between glomerular cells and infiltrating inflammatory cells with respect to RANTES/CCL5 expression likely contributes to a further increase in the number of infiltrating inflammatory cells. The cross talk between glomerular cells and infiltrating inflammatory cells for an increased chemokine release might not only depend on the presence of ICAM-1 receptors and their ligand counterparts, but also might involve other receptor-ligand interactions.

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APPENDIX

Abbrevations used in this article are: a-M/M, rabbit anti-rat M/M antiserum (polyclonal); a-RM, rabbit anti-rat macrophage antiserum (specific); CyP, cyclophosphamide; EMSA, electrophoretic mobility shift assay; GBM, glomerular basement membrane; ICAM-1, intracellular cell adhesion molecule-1; IL-8, interleukin-8; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1 (CCL2); M/M, monocytes/macrophages; NF-κB, nuclear factor-κB; NM, nitrogen mustard; PCNA, proliferating cell nuclear antigen; PMN, polymorphonuclear leukocytes; RANTES, regulated upon activation, normal T cell expressed and secreted (CCL5); SPF, specific-pathogen-free; Thy 1.1-nephritis, glomerulonephritis induced by intravenous injection of a rabbit anti-rat thymocyte antiserum.

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