J Am Soc Nephrol 14: 317–326, 2003

Bacterial CpG-DNA Aggravates Immune Complex Glomerulonephritis: Role of TLR9-Mediated Expression of Chemokines and Chemokine Receptors

HANS-JOACHIM ANDERS,* BERNHARD BANAS,* YVONNE LINDE,* LARS WELLER,* CLEMENS D. COHEN,* MATTHIAS KRETZLER,* STEFAN MARTIN,† VOLKER VIELHAUER,* DETLEF SCHLÖNDORFF,* and HERMANN-JOSEF GRÖNE†

**Nephrological Center, Medical Policlinic, University of Munich, Munich, Germany; †Division of Cellular and Molecular Pathology, German Cancer Research Center, Heidelberg, Germany.*

Abstract. Immune complex glomerulonephritis (GN) often deteriorates during infection with viruses and bacteria that, in contrast to mammals, have DNA that contains many unmethylated CpG motifs. Balb/c mice with horse apoferritin-induced GN (HAF-GN) were treated with either saline, CpG-oligodeoxynucleotides (ODN), or control GpC-ODN. Only CpG-ODN exacerbated HAF-GN with an increase of glomerular macrophages, which was associated with massive albuminuria and increased renal MCP-1/CCL2, RANTES/CCL5, CCR1, CCR2, and CCR5 mRNA expression. CpG-ODN induced a Th1 response as indicated by serum anti-HAF IgG_{2a} titers, mesangial IgG_{2a} deposits, and splenocyte IFN-γ secretion. Messenger RNA for the CpG-DNA receptor Toll-like reeptor 9 (TLR9) was present in kidneys with HAF-GN but not in normal kidneys. The source of TLR9 mRNA in HAF-GN could be infiltrating macrophages or intrinsic renal cells, *e.g.*, mesangial cells; but, *in vitro*, only murine J774 macrophages expressed TLR9. In J774 cells, CpG-ODN induced the chemokines MCP-1/CCL2 and RANTES/CCL5 and the chemokine receptors CCR1 and CCR5. It is concluded that CpG-DNA can aggravate preexisting GN via a shift toward a Th1 response but also by a novel pathway involving TLR9-mediated chemokine and chemokine receptor expression by macrophages, which may contribute to the enhanced glomerular macrophage recruitment and activation. This mechanism may be relevant during infection-triggered exacerbation of human immune-complex GN and other immune-mediated diseases in general.

Bacterial or viral infections often induce flares of various immune and autoimmune syndromes. In the kidney, infections can trigger the onset and exacerbation of immune complex glomerulonephritis (GN) (1,2). During IgA nephropathy, systemic lupus erythematosus, and renal vasculitis, bacterial infections also trigger activity that may induce progressive loss of renal function up to end-stage renal disease (1,3).

The immunostimulatory effects of bacterial or viral molecules such as LPS, lipoproteins, peptidoglycans, and RNA/DNA are recognized through the different members of the MyD88-dependent Toll-like receptor (TLR) family (4). It has recently been found that the stimulatory potential of prokaryotic DNA relates to the presence of the unmethylated CpG-dinucleotide, which occurs at high frequency in prokaryotic in contrast to mammalian DNA (5–8). The unmethylated CpG motif is specifically recognized by TLR9, which leads to activation of NF-κB involving the IRAK and TRAF6 cascade (4,9,10). CpG-DNA-TLR9 interaction stimulates B cells, macrophages, and dendritic cells to secrete cytokines, especially Th1-like cytokines such as IL-12 and IL-18 (4,11). Induction of a Th1-type response with CpG-ODN is considered as a promising approach for vaccination strategies, treatment of infections by intracellular pathogens, or diseases characterized by a predominant Th2-type response (12–16).

However, the immunostimulatory potential of prokaryotic or even hypomethylated self-DNA may also affect underlying chronic inflammatory diseases or autoimmunity (17–20), *e.g.*, immune complex GN, as Th1/Th2 balance, macrophage activity, and B cell function contribute to the pathogenesis of renal immune complex disease (21,22). We therefore hypothesized that CpG-DNA would aggravate a preexisting immune complex GN, a hypothesis confirmed by our results. The worsening of glomerular damage was associated with marked macrophage infiltration, a finding also noted by others using CpG-ODN in various disease models (23–25). Further analysis revealed, as a novel mechanism, that CpG-DNA-TLR9 interaction induced chemokine and chemokine receptor expression in macrophages. We therefore conclude that CpG-DNA can aggravate immune complex GN via multiple pathways, including increased chemokine and chemokine receptor expression vby macrophages supporting further macrophage infiltration into

Received August 19, 2002. Accepted September 24, 2002.
Correspondence to Dr. Detlef Schlöndorff, Medizinische Poliklinik der LMU, Pettenkoferstr. 8a, 80336 München, Germany. Phone: 49-89-51603500; Fax: 49-89-51604439; E-mail: Detlef.Schloendorff@pk-i.med.uni-muenchen.de

1046-6673/1401-0317
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000042169.23931.73

the glomerulus. These data may also have important implications for the understanding of infection-associated exacerbations of autoimmune diseases in general.

Material and Methods

Animals and Experimental Protocol

Female inbred mice of the Balb/c strain, 18 to 21 g, were obtained from Charles River (Sulzfeld, Germany) and were kept in macrolone type III cages under a 12-h light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available *ad libitum*. Mice were sacrificed by cervical dislocation. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities. The following groups were studied. In group 0, mice received 80 μl normal saline once daily, intraperitoneally. In group I, mice were injected with 4 mg of horse-spleen apoferritin (HAF) in 80 μl of 0.1 M sodium chloride (Sigma-Aldrich Chemicals, Steinheim, Germany) once daily intraperitoneally for 14 consecutive days, as described previously (26). Mice of this group received 100 μl of normal saline intraperitoneally eight hours after the HAF-injection on days 7 and 8. In group II, HAF was administered once daily as above. On days 7 and 8, 40 μg of endotoxin-free control phosphothioate GpC-oligonucleotide (5'-TCG ATG A*GC* TTC CTG ATG CT-3'; TIB Molbiol, Berlin, Germany) was given in 100 μl of normal saline intraperitoneally 8 h after the HAF-injection. In group III, HAF daily plus 40 μg endotoxin-free CpG-ODN (5'-TCG ATG A*CG* TTC CTG ATG CT-3', TIB Molbiol) in 100 μl of saline intraperitoneally on days 7 and 8 as in group II.

To assess the renal distribution of injected CpG-ODN, 40 μg of 3'-rhodamine-labeled CpG-ODN (TIB Molbiol) were injected intraperitoneally into Balb/c mice with and without HAF-GN on day 14. Serum samples and renal tissue were collected 4 h later and subjected to further analysis as described below.

Evaluation of Immune Complex Glomerulonephritis

Blood samples were collected from each animal at the end of the study by bleeding from the retro-orbital venous plexus under general anesthesia with inhaled ether. Spot urine samples were collected from each animal at the end of the study, and urine albumin concentration was measured using a commercial mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). The immune response to the injected antigen was assessed by measuring anti-HAF titers by an ELISA method as described previously (26). The following antibodies were used as detection antibodies: total mIg, P260 (Dako, Hamburg, Germany); $mIgG_1$ (Pharmingen, Hamburg, Germany); $mIgG_{2a}$ and mIgM (Dianova, Hamburg, Germany).

Light and Electron Microscopical Evaluation

From each mouse, the left kidney was used for histologic assessment. Two-micrometer-thick paraffin-embedded sections were cut and stained with periodic acid-Schiff reagent. The severity of the renal lesions was graded by two blinded investigators using the indices for activity and chronicity as described for human proliferative lupus nephritis (27). A small piece of cortical tissue from the lower kidney pole was fixed in glutaraldehyde and embedded in araldite for EM analysis as described (26).

Immunofluorescence

The distribution of rhodamine-labeled ODN was assessed on frozen sections after acetone fixation. HAF deposits were detected on frozen sections by a FITC-conjugated anti-horse ferritin antibody (1:100; Jackson ImmunoResearch, West Grove, PA) and complement factor-3 (C3) by a FITC-conjugated anti mouse C3 antibody (ICN Pharmaceuticals, Frankfurt, Germany). Thirty glomeruli were counted from each section, and signals were assessed by a semiquantitative score: 0 = no signal, 1 = low signal intensity, 2 = moderate signal, 3 = strong signal.

Immunohistology

Paraffin-embedded sections were deparaffinized and microwave-treated for antigen retrieval. For staining of immunoglobulins, acetone-fixed frozen sections were used as described (26). The following rat and rabbit antibodies were used as primary antibodies: anti-Mac2 (macrophages, Pharmingen, 1:50), anti-CD3 (lymphocytes, Pharmingen, 1:100), anti-Ki-67 (cell proliferation, Dianova, rabbit, 1:100), $mIgG_1$ (Pharmingen, 1:100), $mIgG_{2a}$ (Dianova, 1:100), and mIgM (Dianova 1:100). Glomerular cells were counted in 15 cortical glomeruli per section. Scoring of mesangial IgG depositis was performed as described above.

Measurement of Interferon (IFN)-γ Production by Splenocytes

Spleens were removed under aseptic conditions and placed in RPMI 1640 10% fetal calf serum on ice. After manual dissection, centrifugation, and an additional wash step, single cell suspensions (4×10^6 cells/ml) were incubated in RPMI 1640 (Biochrom KG, Berlin, Germany) plus 10% fetal calf serum and 10 μg/ml phytohemagglutinin at 37°C, 5% CO_2. Culture supernatants were removed after 48 h, and IFN-γ concentrations were determined with an ELISA kit (Pharmingen).

RNase Protection Assay

From each animal, the right kidney was snap frozen in liquid nitrogen and total RNA was prepared as described (26). Multi-probe template sets for mouse CC chemokines (mCK-5) and mouse CC chemokine receptors (mCR-5, Pharmingen, San Diego, CA) were performed as described (26). Finally, gels were exposed on phosphor screens of a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Studies with Macrophage-Like J774 Cells and Mesangial Cells

For analysis of potential effects of ODN on murine macrophages or intrinsic glomerular cells, the following cell lines were used. J774 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 containing 1mM HEPES, 10% heat-inactivated bovine serum, and 1% penicillin-streptomycin. A murine mesangial cell line was maintained in Dulbecco modified Eagle's medium (DMEM; Biochrom KG, Berlin, Germany) supplemented with 5% bovine serum (Serum Supreme; BioWhittaker, Walkersville, MD) and 1% penicillin-streptomycin 100 U/ml and 100 μg/ml (Biochrom KG, Berlin, Germany) as described (28). J774.1 cells and mesangial cells were incubated for 6 and 24 h with 3 μM of CpG-ODN, GpC-ODN, or standard medium without supplements. After stimulation, cells were harvested by trypsinization and total RNA was prepared as above.

PCR for TLR9

Total RNA was treated with DNase I (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using Superscript reverse transcriptase (Qiagen). Negative control samples were prepared by performing the latter reaction in the absence of reverse transcriptase (RT- control). cDNA was analyzed by performing PCR using an initial cycle of 62° for 45 s and 94°C for 45 s, followed by 30 cycles of 94°C and 72°C for 45 s each. The following primers were used to detect the corresponding murine gene products: TLR9 (f, 5-AGG CTG TCA ATG GCT CTC AGT T-3; r, 5-TGA ACG ATT TCC AGT GGT ACA AGT-3); β-actin (f, 5-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3; r, 5-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3). Total spleen cDNA was used as a positive control for TLR9 expression.

Real-Time Quantitative RT-PCR

RNA preparation and real-time RT-PCR was performed as described (26). Controls consisting of ddH_2O were negative for target and the housekeeper 16S rRNA. The following oligonucleotide primers (300 nM) and probes (100 nM) were used. Murine CCR1: sense *5*'-TTAGCTTCCATGCCTGCCTTATA-3', antisense 5'-TCCACTGCTTCAGGCTCTTGT-3', internal fluorescence-labeled probe (FAM) 5'-ACTCACCGTACCTGTAGCCCTCATTTCCC-3'; murine CCR2: sense 5'-CCTTGGGAATGAGTAACTGTGTGA-3'; antisense 5'-ACAAAGGCATAAATG-ACAGGATTAATG-3', FAM 5'-TGACAAGCACTTAGACCAGGCCATGCA-3'; murine CCR5: sense 5'-CAAGACAATCCTGATCGTGCAA-3', antisense 5'-TCCTACTCCCAAGCTGCATA-GAA-3', FAM 5'-TCTATACCCGATCCACAGGAG-AACATGAAGTTT-3'; CCR5 specificity of primers and probe were tested on CCR plasmids. The murine 16S rRNA probe was obtained as predeveloped probe from PE Biosystems, Weiterstadt, Germany.

Statistical Analyses

Data are expressed as mean ± SD. Comparison of groups was performed using univariate ANOVA, and *post hoc* Bonferroni correction was used for multiple comparisons. Paired Student's *t* test was used for the comparison of single groups (IFN-γ data). A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Induction of Immune Complex GN

In Balb/c mice, daily HAF injections induced high serum titers of anti-HAF IgG and IgM (Table 1). Immune deposits consisting of HAF and IgG as well as complement factor C3 were noted in a mesangial staining pattern in almost all glomeruli (Figure 1, C3 staining not shown). HAF-injected mice had diffuse GN with mesangial expansion and hypercellularity (Figure 2). Glomerular cell proliferation was noted by an increase of Ki-67–positive cells (Table 1; Figure 2). Proliferating cells were located to areas of mesangial expansion and not to endothelial cells or podocytes and mainly represented mesangial cells and macrophages (Figure 2). Glomerular infiltration of Mac2–positive macrophages was prominent compared with the mild increase of glomerular CD3 T cells (latter not shown, Table 1; Figure 3). Consistent with the histology of GN, HAF-injected Balb/c mice developed albuminuria (HAF *versus* saline, 83 ± 29 *versus* 16 ± 4 μg albumin/ml; $P < 0.05$; Figure 3).

Distribution Pattern of CpG-ODN in Healthy Mice and in HAF-GN

Four hours after intraperitoneal injection of 3'-rhodamine-labeled CpG-ODN into healthy mice of the Balb/c strain, these

Table 1. Serum, urinary, and histologic findings in HAF-GN[a]

Groups	Saline	HAF + Saline	HAF + GpC-ODN	HAF + CpG-ODN
Humoral response				
serum titers				
anti-HAF IgM	—	3413 ± 1182	3413 ± 1182	5461 ± 2896
anti-HAF IgG	—	2304 ± 1288	2560 ± 1024	2253 ± 1122
anti-HAF IgG_1	—	922 ± 229	896 ± 256	937 ± 600
anti-HAF IgG_{2a}	—	768 ± 296	896 ± 256	2219 ± 1122[b]
IgG_{2a}/IgG_1 ratio	—	0.9 ± 0.3	1.0 ± 0.0	2.7 ± 1.1[b]
glomerular deposits				
HAF	—	2.1 ± 0.3	2.0 ± 0.3	2.0 ± 0.2
C3	—	2.0 ± 0.3	1.9 ± 0.3	2.0 ± 0.5
IgG_1	—	1.8 ± 0.2	1.7 ± 0.2	1.6 ± 0.4[b]
IgG_{2a}	—	0.8 ± 0.3	1.0 ± 0.3	1.9 ± 0.4[b]
IgG_{2a}/IgG_1 ratio	—	0.5 ± 0.2	0.6 ± 0.2	1.3 ± 0.6[b]
spleen cytokines				
IFN-γ (pg/ml)	ND	50.6 ± 29.4	53.1 ± 56.5	403.9 ± 230.9[b]
Cellular response (cells/glom.)				
Mac2+	0.5 ± 0.2[b]	4.0 ± 0.4	3.4 ± 0.5	7.0 ± 0.6[b]
CD3+	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Ki-67+	0.6 ± 0.2[b]	1.6 ± 0.3	1.8 ± 0.3	4.0 ± 1.0[b]

[a] —, not detected; ND, not done.
[b] $P < 0.05$ group saline, HAF + GpC + ODN; and HAF + CpG − ODN *versus* group HAF + saline.

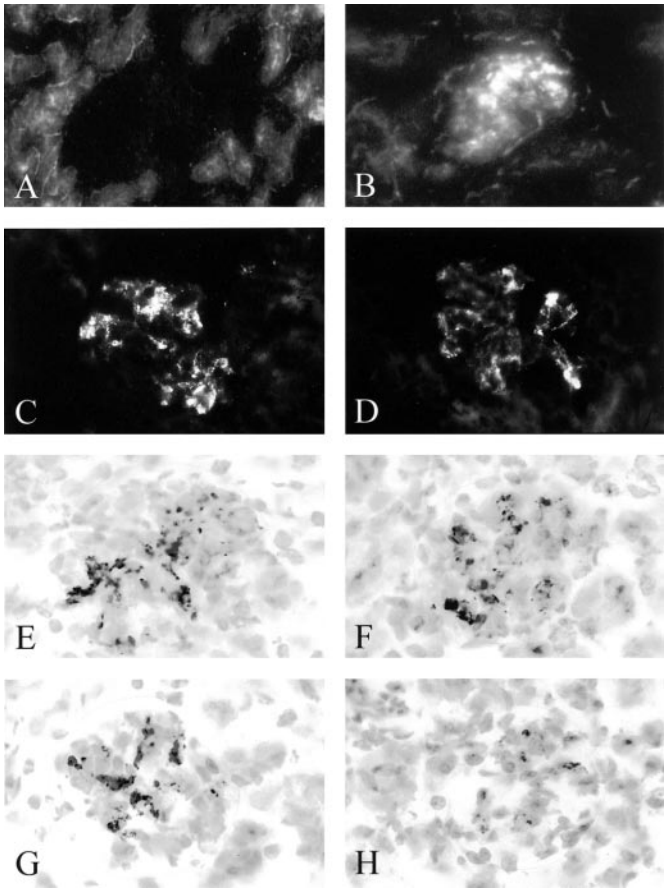

Figure 1. Renal CpG-oligodeoxynucleotides (ODN) distribution and mesangial immune deposits. When injected into normal mice, 3'-rhodamine-labeled CpG-ODN localized to proximal tubular cells but not to glomeruli 4 h after intraperitoneal injection (A). In contrast, in mice with horse apoferritin-induced GN (HAF-GN), 3'-rhodamin-labeled CpG-ODN also accumulated in glomeruli with a capillary and mesangial staining pattern (B). Injection of 3'-rhodamine alone did not result in renal staining, indicating that the glomerular and tubular uptake was independent of the fluorochrome itself (not shown). In Balb/c mice with HAF-GN, HAF deposits were found with a mesangial staining pattern in all groups and to comparable extent (saline: not shown, GpC-ODN: C, CpG-ODN: D, FITC-labeled anti horse ferritin, ×1000). In Balb/c mice, mesangial IgG subclass deposits were characterized by immunohistochemistry; IgG_1 was found to a similar extent in mice treated with either GpC- (E) or CpG-ODN (F). The amount of mesangial IgG_{2a} deposits was increased with CpG-ODN treatment (H) *versus* GpC-ODN-treated controls (G, counterstained with hemalaun, ×1000).

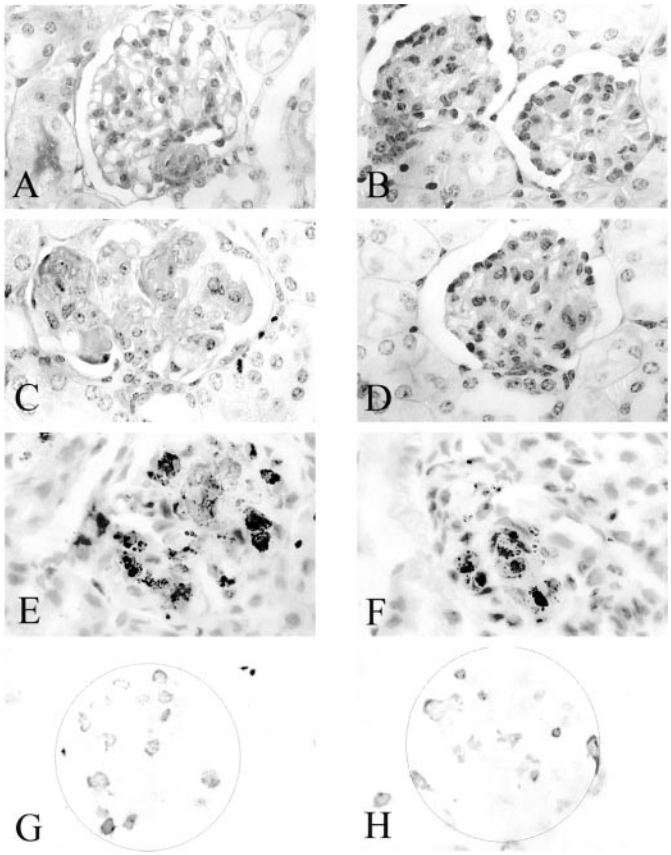

Figure 2. Histopathology of HAF-GN in Balb/c mice (A through D). Compared with saline-injected controls (A), HAF-injected mice developed diffuse mesangial-proliferative GN (B). Treatment with CpG-ODN resulted in aggravation of mesangial hypercellularity, capillary thrombosis, and focal glomerulosclerosis (C). Treatment with the control GpC-ODN had no effect on HAF-GN (D). (E through H) Immunohistochemistry of Balb/c mice with HAF + CpG-ODN (E and G) and mice with HAF + saline (F and H). CpG-ODN induced a marked increase of Mac2-positive macrophages in the glomerulus (E) compared with untreated controls with HAF-GN (F, counterstained with hemalaun, ×1000). The amount of proliferating (K_i-67–positive) cells in the glomerulus also increased with CpG-ODN (G) compared with untreated controls with HAF-GN (H, ×1000).

accumulated in proximal tubular cells but not glomeruli as assessed by immunofluorescence (Figure 1). In contrast in HAF-GN, CpG-ODN also localized to glomeruli in a capillary and mesangial staining pattern (Figure 1). ODN-free 3'-rhodamine alone did not accumulate in the kidney, indicating that the staining of rhodamine was due to tubular uptake of filtered ODN in the control mice and to deposition with the immune complexes in mice HAF-GN (Figure 1). Normal Balb/c mice given two injections of 40 μg of CpG-ODN intraperitoneally on days 7 and 8 had no glomerular or tubulointerstitial abnormalities on day 14 (not shown).

CpG-ODN but Not Control GpC-ODN Aggravate HAF-GN

In mice with HAF-GN, two injections with CpG-ODN markedly aggravated albuminuria (Figure 3). This finding correlated with an aggravation of histopathologic markers of glomerulonephritis, such as increased mesangial expansion and glomerular proliferation (as judged by Ki-67–positive cells), capillary thrombosis, and glomerulosclerosis (Table 1; Figure 2). Renal damage was restricted to glomeruli, and changes of the tubulointerstitium were not observed. The extent of renal damage is illustrated by an increase of the activity and chronicity index for proliferative GN. In contrast, injection of the control GpC-ODN in HAF-GN mice did not aggravate the

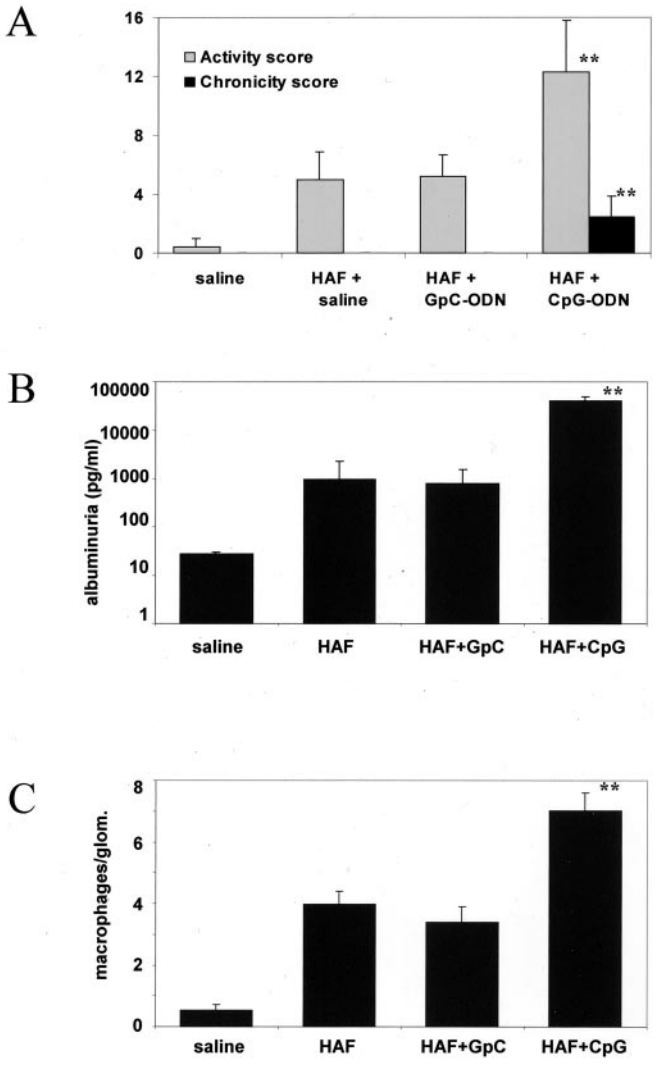

Figure 3. Score for renal damage, albuminuria, and glomerular macrophages. (A) The scores for activity and chronicity in proliferative GN were used to assess the extent of renal damage in HAF-GN. Treatment with GpG-ODN did not affect the parameters of disease activity and chronicity compared with saline-injected mice with HAF. In contrast, CpG-ODN aggravated both indices as compared with GpC-ODN-treated mice ($P = 0.0002$ for activity index, $P = 0.0017$ for chronicity index, $n = 5$ to 7 mice in each group). (B) Albuminuria was assessed by ELISA and is illustrated in a semilogarithmic scale for all groups. CpG-ODN induced massive albuminuria compared with GpC-ODN–treated or saline-treated mice with HAF-GN ($P < 0.0001$). (C) The amount of glomerular macrophages was assessed by immunohistochemistry using a Mac2-specific antibody. CpG-ODN increased the amount of glomerular macrophages compared with GpC-ODN–treated or saline-treated mice with HAF-GN ($P < 0.05$; $n = 5$ to 7 mice in each group).

glomerular disease (Figure 3). Mesangial HAF and C3 staining was not affected by CpG-ODN treatment.

Ultrastructurally in glomeruli of HAF-GN mice given the CpG-ODN, electron-dense deposits of varying size could be seen in the mesangium, were more numerous than in HAF-GN mice given the GpC-ODN, and could also be observed in a subendothelial distribution in glomerular capillaries (Figure 4). In HAF-GN mice injected with control-ODN, capillaries were frequently patent but with CpG-ODN macrophages with lysosomes, and fat droplets obstructed the majority of capillaries and were also present in large amounts within the mesangium (Figure 4). CpG-ODN-treated mice had diffuse podocyte foot process effacement compared with GpC-ODN–treated mice that showed normal to plumb podocyte foot processes (Figure 4).

CpG-ODN increased the amount of glomerular Mac2–positive macrophages but not of CD3-positive lymphocytes (CpG-HAF, 9.9 ± 0.3 *versus* HAF, 6.1 ± 0.6 macrophages/glom; $P < 0.05$; Figure 2). Injection with the GpC-ODN in HAF-GN did not affect histopathologic findings and albuminuria compared with saline-injected HAF-GN mice (Table 1).

CpG-ODN Induce an Immune Response of the Th1-Type

To characterize the Th1/Th2 balance during HAF-GN, we measured serum titers of HAF-specific IgG_1 and IgG_{2a} as a marker of B cell response. The IgG subclasses within mesangial immune deposits were also assessed by immunohistochemistry. In addition, splenocyte IFN-γ secretion was measured as a marker of T cell polarization. CpG-ODN–treated mice had an increase of HAF-specific serum IgG_{2a} and of mesangial IgG_{2a} deposits as compared with GpC-ODN and saline controls (Table 1; Figure 1). In contrast, the serum titers

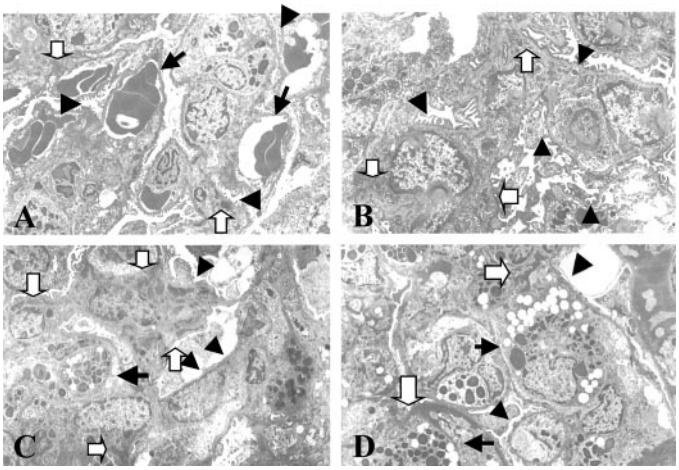

Figure 4. Electron microscopy of HAF-GN. Glomeruli of mice with HAF-GN (A and B) that received GpC-ODN injections at days 6 and 7 showed open capillaries containing erythrocytes (black arrows in A), mesangial hypercellularity, and increase of mesangial matrix with hyperdense immune deposits (open arrows in A through D). Normal podocyte foot processes morphology was maintained (arrowheads in B). In HAF-GN mice injected with CpG-ODN (C and D), the ultrastructural glomerular abnormalities were severely aggravated. Most capillaries were obliterated and filled with macrophages that contained many lipid droplets and electron-dense endosomes as a marker of macrophage activation (black arrows in C). The mesangium of mice with HAF-GN (open arrows in C and D) contained electron-dense deposits (A and B). The visceral epithelium showed marked podocyte foot process effacement (arrowheads in C and D).

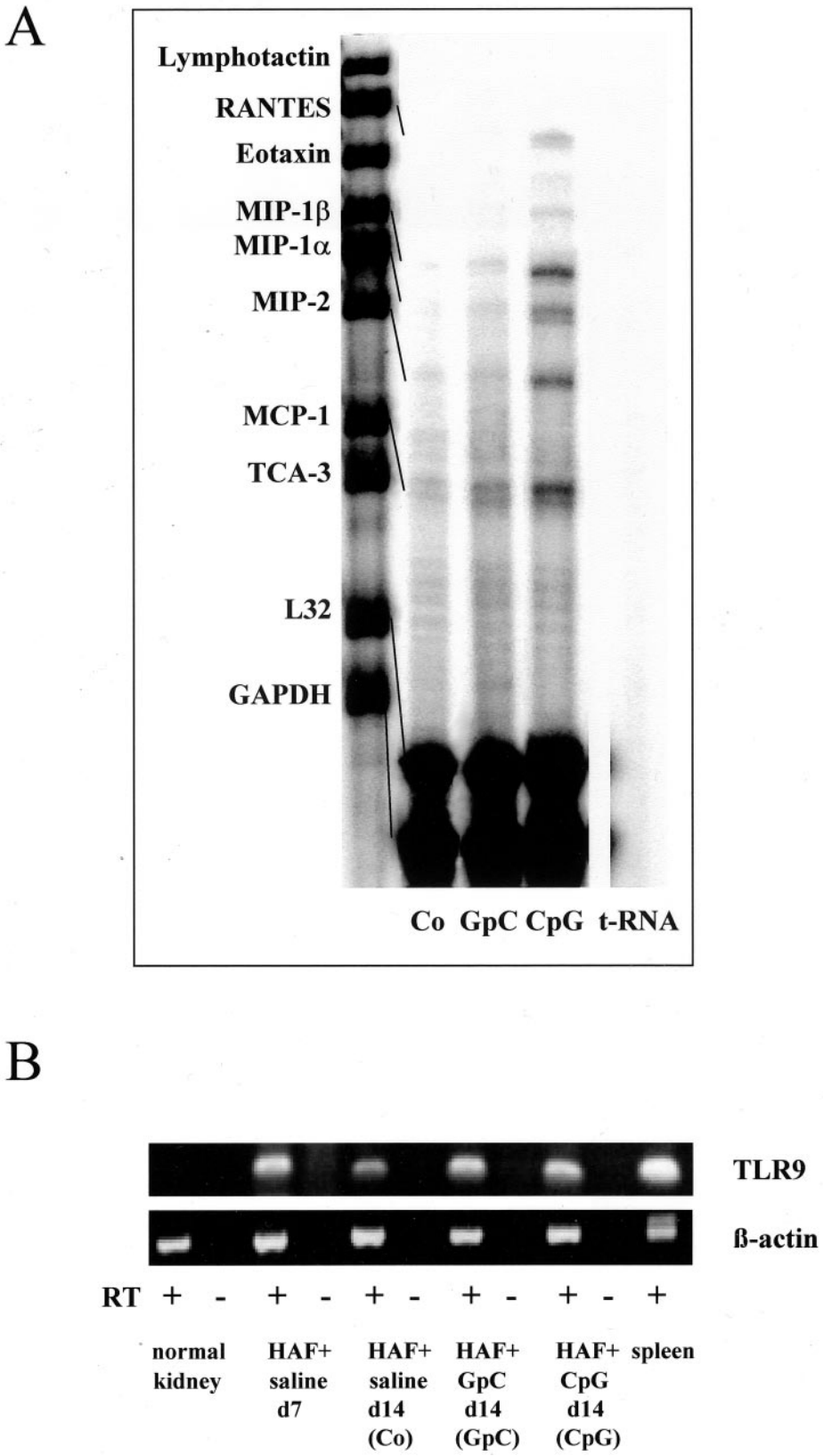

Figure 5. Renal mRNA expression of CC-chemokines and TLR9 during HAF-GN. (A) Chemokine expression was analyzed by multiprobe RNase protection assay using total renal RNA at day 14 of HAF-GN. The unprotected probe is shown on the right, and the protected fragments are indicated on the left. At 14 d, renal tissue of mice with HAF-GN did not express RANTES, Eotaxin, MIP-1β, or TCA-3 and had low mRNA expression levels for MIP-1α, MIP-2, and MCP-1. Treatment with CpG-ODN but not with GpC-ODN induced renal RANTES, MIP-1α, MIP-2, MIP-1β, and MCP-1 mRNA expression. The illustration is representative for assays on tissue from three mice of each group. (B) Chemokine

of total HAF-specific IgG_1 and mesangial IgG_1 deposits were comparable in all groups of mice with HAF-GN. CpG-ODN also increased IFN-γ production of isolated splenocytes compared with GpC-ODN and saline controls (HAF+CpG, 404 ± 231 pg/ml *versus* HAF+GpC, 52 ± 57 pg/ml; $P < 0.002$). GpC-ODN did not affect total serum IgG titers compared with saline-injected HAF-GN mice. In contrast, the trend toward increased serum IgM titers in CpG-ODN–treated HAF-GN mice did not reach statistical significance (Table 1). Together, these data indicate that CpG-ODN specifically induced an immune response of the Th1-type in the Balb/c mice with HAF-GN.

CpG-ODN Treatment Stimulates Renal Chemokine and Chemokine Receptor Expression

To examine whether the increased amount of glomerular macrophages in Balb/c mice correlated with local chemokine expression, we performed RNase protection assays with mRNA isolated from the affected kidneys. At 14 d, HAF-GN kidneys contained low levels of CC-chemokine mRNAs for CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/MIP-2 as reported previously (Figure 5A; reference 26). Treatment with CpG-ODN significantly increased renal chemokine mRNA expression of the chemokines CCL5/RANTES, CCL3/MIP-1α, CCL4/MIP-1β, CXCL1/MIP-2, and CCL2/MCP-1. GpC-ODN had no significant effect on chemokine mRNA expression in kidneys with HAF-GN (Figure 5A).

Renal chemokine receptor expression was determined by quantitative real-time RT-PCR. At 14 d, HAF-GN kidneys contained low levels of mRNAs for CCR1, CCR2, and CCR5 (Figure 5B). Treatment with CpG-ODN significantly increased renal mRNA expression of CCR1 and CCR5. For CCR2, the increase did not reach statistical significance. GpC-ODN had no significant effect on chemokine receptor mRNA expression in kidneys with HAF-GN (Figure 5B).

TLR9 Is Expressed in the Kidney with HAF-GN but Not in Normal Mice

CpG-DNA requires interaction with TLR9 to mediate its stimulatory effects. RT-PCR for TLR9 using total kidney RNA from mice of the various groups revealed that TLR9 was not expressed in renal tissue from healthy mice (Figure 5C). At day 7, of HAF-GN, the time just before the first administration of CpG-DNA, kidneys of Balb/c mice with HAF-GN expressed TLR9. At day 14, kidneys from all groups of mice with HAF-GN also expressed the TLR9 irrespective of ODN injections (Figure 5C). Thus induction of HAF-GN resulted in a positive signal for TLR9 in the affected kidneys irrespective of CpG-ODN administration.

Macrophages but Not Mesangial Cells Express the CpG-DNA Receptor TLR9

In HAF-GN, the glomerular hypercellularity consisted of infiltrating macrophages and proliferation of mesangial cells. To evaluate which glomerular cell type contributes to the TLR9 expression during HAF-GN, we studied the expression of TLR9 in established cell lines of murine macrophages and mesangial cells. RNA isolates of J774 cells and mesangial cells. J774 cells constitutively expressed TLR9 as determined by RT-PCR (Figure 6). In contrast, TLR9 was not detected in mesangial cells, indicating that the TLR9 expression seen in kidney isolates probably originated from infiltrating macrophages (Figure 6).

CpG-ODN Stimulate Chemokine and CCR1 and CCR5 mRNA Expression in J774 Cells In Vitro

To examine whether macrophages contributed to the CpG-induced chemokine expression of renal tissue, we performed *in vitro* studies with J774 cells and used TLR9-negative mesangial cells as a control. Multiprobe RNase protection assays were used to quantify chemokine and chemokine receptor mRNA expression. Unstimulated J774 cells showed basal mRNA levels of CCL3/MIP-1α, CCL4/MIP-1β, CXCL1/MIP-2, and CCL2/MCP-1 (Figure 7). Incubation with CpG-ODN for 6 and 24 h markedly increased the mRNA expression of all of the above chemokines and in addition CCL5/RANTES (Figure 7). GpC-ODN did not consistently influence chemokine mRNA levels in J774 cells. Under basal culture conditions, J774 cells showed constitutive levels of mRNA for the chemokine receptors CCR1 and CCR5, which were increased by 2.3-fold and 2.9-fold, respectively, in the presence of CpG-ODN (Figure 7). Incubation with the GpC-ODN had no effect on chemokine receptor expression.

In contrast, TLR9 negative murine mesangial cells did not reveal any differences in their low basal expression of CCL5/RANTES and CCL2/MCP-1 after GpC- or CpG-ODN exposure (data not shown). Mesangial cell levels of mRNA for chemokine receptors were undetectable for CCR1, CCR2, and CCR5. Neither CpG- nor GpC-ODN induced mesangial cell mRNA levels for these receptors (not shown).

Discussion

Toll-like receptors and specifically TLR9 as pattern recognition receptors have been implicated in a link between the

receptor expression was analyzed by real-time RT-PCR using total renal RNA at day 14 of HAF-GN. The expression in saline-treated mice with HAF-GN is set as 1. At 14 d, renal tissue of mice with HAF-GN did express only small amounts of chemokine receptor CCR1, CCR2, and CCR5 mRNA. Treatment with CpG-ODN but not with GpC-ODN induced renal CCR1 ($P = 0.04$) and CCR5 ($P < 0.04$) mRNA expression. For CCR2, the increase did not reach statistical significance. (C) Renal TLR9 expression was analyzed by RT-PCR. In contrast to normal kidneys, those of all groups with HAF-GN expressed mRNA for TLR9 in two sets of experiments. Murine total spleen cDNA served as positive control for TLR9 expression and respective RT^+ and RT^- probes and β-actin expression serves as a control of PCR performance.

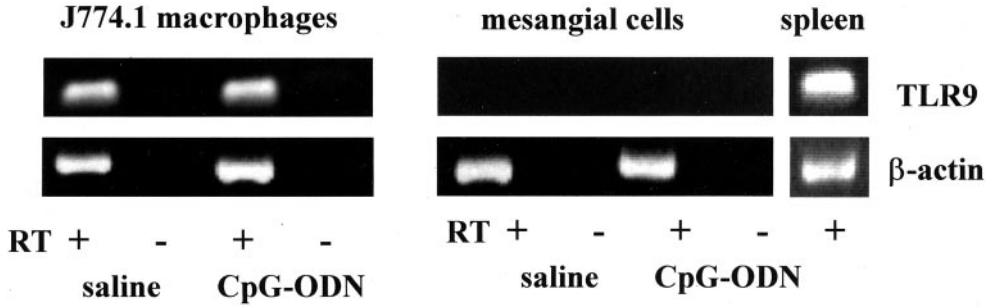

Figure 6. TLR9 expression in CpG-stimulated J774.1 macrophages and mesangial cells. TLR9 expression was analyzed by RT-PCR in unstimulated and CpG-ODN–stimulated cells. In three independent experiments, murine J774.1 macrophages expressed TLR9 constitutively and after incubation with CpG-ODN. MC did not express TLR9 constitutively or after stimulation with CpG-ODN. Murine total spleen cDNA served as positive control for TLR9 expression, and respective β-actin expression is presented as a control of PCR performance. The controls without reverse transcription are shown as RT^-.

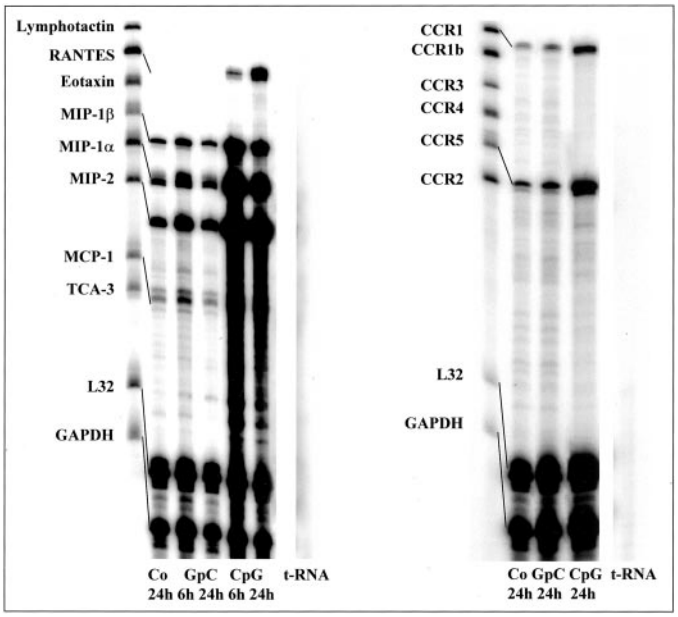

Figure 7. Expression of chemokine (A) and chemokine receptor (B) mRNA by murine J774.1 macrophages. Chemokine (A) and chemokine receptor (B) mRNA expression were analyzed by multiprobe RNase protection assays. The unprotected probe is shown on the right, and the protected fragments are indicated on the left. (A) After 6 and 24 h, CpG-ODN markedly induced RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA expression. The GpC-ODN had no effect on the expression of chemokines compared with saline controls (Co). (B) After 24 h, CpG-ODN induced the expression of CCR1 and CCR5 mRNA compared with CCR2 mRNA levels in saline or GpC-ODN–treated cells.

innate and adaptive immune system by favoring a Th1 immune response and enhancing autoantibody production (4,19,29). We used CpG-ODN in HAF-GN as a model of intercurrent delivery of unmethylated CpG-DNA during an underlying immune complex GN. At day 7, immune complex disease is already present in this model, and two injections of CpG-ODN severely aggravated glomerular damage and proteinuria at day 14, which was associated with an increase of glomerular mesangial proliferation and macrophage infiltration and an enhanced Th1 response. Furthermore, CpG administration resulted in enhanced mRNA levels for several chemokines and chemokine receptors in kidneys with HAF-GN. *In vitro* studies demonstrated that the CpG-DNA receptor TLR9 was expressed by J774 macrophages but not by mesangial cells. In J774 cells, CpG-ODN induced several CC-chemokines and their receptors, indicating that the increased renal chemokine expression seen in CpG-ODN-treated mice with HAF-GN is mainly derived from infiltrating macrophages. Together, these data indicate that unmethylated CpG-DNA can aggravate immune complex GN by enhancing a Th1 response and, as a novel mechanism, by increasing glomerular macrophage recruitment via inducing the selective expression of chemokines and chemokine receptors on macrophages. This enhanced TLR9-mediated chemokine receptor expression together with other CpG-ODN-mediated mechanisms may be relevant for infection-triggered exacerbation of immune- and autoimmune-mediated renal and extrarenal diseases.

CpG-ODN Aggravate HAF-GN in Balb/c Mice in a Sequence-Specific Manner

Our observation that the aggravation of HAF-GN depends on the CpG dinucleotide sequence underlines the importance of this motif for the immunostimulatory actions of DNA, which was identified as the trigger sequence in 1995 by Krieg *et al.* (6). CpG-DNA mediates its effects exclusively via TLR9 (10). Although there are obvious similarities to the activation of immune cells by prokaryotic LPS, lipoproteins, and peptidoglycans, these are mediated by other members of the TLR family (4,30,31). In contrast to the toxic effects of the latter antigens on naïve animals or humans, our present studies and previous ones by others demonstrate that CpG-DNA alone does not affect healthy mice (10). In extension of the concept of an innate, nonspecific (Th1) response to unmethylated CpG-DNA, the recognition of CpG-DNA-IgG complexes by crosslinking of B cell surface IgM receptors and TLR9 has been shown to stimulate DNA-specific IgG and IgM secretion as a link to adaptive immunity (1,32). Although the activation of both innate and adaptive systems may support pathogen

clearance (5), both responses may also lead to unfavorable effects in autoimmunity and chronic inflammatory disease (4). The clinical observation that infections can exacerbate chronic inflammation in humans, and recent findings from *in vivo* models support this hypothesis. For example, CpG-ODN aggravated rat experimental allergic encephalomyelitis and collagen-induced arthritis in DBA/1 LacJ mice, two disease models that, as HAF-GN, rely on antigen-induced immune responses (15,33).

CpG-ODN Induce an Immune Response of the Th1-Type during HAF-GN

CpG-ODN are known stimulators of an Th1-type immune response by induction of IL-12 and IFN-γ that direct T cell differentiation and switch of IgG subclasses (11). This type of immune response is a major strategy of the innate immune system to direct acquired immune responses to control extracellular and intracellular pathogens (15,16,29). However, induction of a Th1 response may significantly alter the course of underlying inflammatory disease states toward either resolution or progression, depending on the disease. For example, CpG-ODN-induced Th1-shift correlated with a marked improvement of ragweed-induced asthma in Balb/c mice (34) and, in contrast, aggravated collagen-induced arthritis in DBA/1 LacJ mice (18). Most of the data on the role of the Th1/Th2 balance in renal disease have been derived from the models of nephrotoxic serum nephritis and lupus nephritis in $MRL^{lpr/lpr}$ mice (20). In Balb/c mice prone to a predominant Th2 response, administration of the Th1 cytokine IL-12 induced marked glomerular crescent formation during nephrotoxic serum nephritis (35). Similarly, daily administration of IL-12 to $MRL^{lpr/lpr}$ mice aggravated glomerular lesions and proteinuria (36). In our experiments with HAF-GN in Balb/c mice, the administration of CpG-DNA induced a Th1-type immune response as demonstrated by increased IFN-γ secretion by splenocytes and an IgG subclass switch toward enhanced IgG_{2a} levels. We therefore conclude from our data that one mechanism by which CpG-DNA can aggravate the course of preexisting immune complex GN would be by favoring an immune response of the Th1-type. The CpG-ODN could be of infectious or endogenous origin, *e.g.*, in lupus nephritis. Thus this mechanism may be relevant to both immune and autoimmune-associated glomerular diseases.

CpG-ODN Induce the Infiltration and Activation of Macrophages in the Glomerulus

CpG-ODN have been shown to induce macrophage activation and recruitment to the site of injection in a variety of animal models (23,24). CpG-ODN given to sensitized SCID beige mice lacking T cells, B cells, and NK cells induced lethal toxic shock via macrophage-derived TNF-α secretion (8). CpG-ODN activated macrophages via TLR9 to secrete a variety of proinflammatory mediators such as IL-1, IL-12, TNF-α, CD80, and CD86 as well as CC-chemokines (6,10,11,37,38). HAF-GN is a model that largely depends on glomerular macrophage accumulation (26). We observed that CpG-ODN administration aggravated renal damage and albuminuria in HAF-GN, which was associated with increased amounts of glomerular macrophages. We therefore questioned whether CpG-ODN altered local chemokine production. In fact, we found enhanced mRNA levels for several CC-chemokines specifically after CpG-ODN administration in kidneys with HAF-GN. Furthermore, while normal kidneys had undetectable levels of mRNA for TLR9, all kidneys with HAF-GN showed positive levels of TLR9 mRNA. As HAF-GN is characterized by mesangial cell proliferation and macrophage infiltration, we examined these cells as potential sources of mRNA for TLR9, CC-chemokines, and chemokine receptors. Using established cell lines of murine macrophages and mesangial cells, we found that J774 cells but not mesangial cells expressed the CpG-DNA receptor TLR9. In addition in J774 cells but not in mesangial cells, CpG-ODN markedly induce the mRNA expression of CC-chemokines and, as a novel finding, the respective chemokine receptors CCR1 and CCR5. We have previously demonstrated the role of the glomerular expression of CCL5/RANTES for the infiltration of CCR1- and CCR5-positive macrophages and lymphocytes in HAF-GN (26,39). Thus the CpG-ODN-induced increase of glomerular macrophages could be a result of CpG-ODN-induced enhancement of chemokine and chemokine receptor expression, especially macrophages with an increase of an intraglomerular amplification loop. In this context, it is of special interest that labeled CpG-ODN colocalize with the glomerular immune deposits in the mice with HAF-GN, whereas no glomerular localization of CpG-ODN occurs in normal kidneys. The colocalization of the immune deposits and the injected CpG-ODN could even indicate a local mechanism of macrophage activation in the glomeruli, a hypothesis deserving further studies. Taken together CpG-ODN triggered chemokine and chemokine receptor expression on macrophages, which results in increased macrophage infiltration into the glomerulus, contributing to further glomerular damage and proteinuria during GN (21).

In summary, unmethylated CpG-ODN aggravated immune complex GN. The immunostimulatory and TLR9-mediated mechanisms of CpG-DNA include the induction of a Th1 response and, as a novel mechanism, the selective induction of chemokine and chemokine receptor expression on macrophages, contributing to their enhanced recruitment to the site of injury. On the basis of these findings, we speculate that bacterial DNA containing the unmethylated CpG motif released during infection or autoimmune diseases may represent a formerly unrecognized trigger for flares in immune-mediated forms of GN, thus contributing to progressive renal dysfunction.

Acknowledgments

The work was supported by grants from the Wilhelm-Sander Foundation, the German Kidney Foundation, and the Deutsche Forschungsgemeinschaft (LU 612/4-1) to HJA, by the Faculty of Medicine of the University of Munich to VV and BB, and by the Deutsche Forschungsgemeinschaft (SFB 405.B10) to HJG and (BA213711) to BB. Parts of the manuscript were prepared as medical thesis at the Faculty of Medicine of the University of Munich by LW.

References

1. Hricik DE, Chung-Park M, Sedor JR: Glomerulonephritis. *N Engl J Med* 339: 888–899, 1998
2. Karkar AM, Rees AJ: Influence of endotoxin contamination on anti-GBM antibody induced glomerular injury in rats. *Kidney Int* 52: 1579–1583, 1997
3. Majumdar A, Chowdhary S, Ferreira MA, Hammond LA, Lipkin GW, Littler WA: Renal pathological findings in infective endocarditis. *Nephrol Dial Transplant* 15: 1782–1787, 2000
4. Akira S, Takeda K, Kaisho T: Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nat Immunol* 2: 675–680, 2001
5. Klinman DM, Verthelyi D, Takeshita F, Ishii KJ: Immune recognition of foreign DNA: A cure for bioterrorism? *Immunity* 11: 123–129, 1999
6. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM: CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374: 546–549, 1995
7. Jones PA, Takai D: The role of DNA methylation in mammalian epigenetics. *Science* 293: 1068–1070, 2001
8. Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, Wagner H: Bacterial DNA causes septic shock. *Nature* 386: 336–337, 1997
9. Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H: Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J Exp Med* 192: 595–600, 2000
10. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S: A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745, 2000
11. Heeg K, Zimmermann S: CpG DNA as a Th1 trigger. *Int Arch Allergy Immunol* 121: 87–97, 2000
12. Krieg AM, Davis HL: Enhancing vaccines with immune stimulatory CpG DNA. *Curr Opin Mol Ther* 3: 15–24, 2001
13. Krieg AM, Kline JN: Immune effects and therapeutic applications of CpG motifs in bacterial DNA. *Immunopharmacology* 48: 303–305, 2000
14. Kline JN, Waldschmidt TJ, Businga TR, Lemish JE, Weinstock JV, Thorne PS, Krieg AM: Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 160: 2555–2559, 1998
15. Wagner H: Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv Immunol* 73: 329–368, 1999
16. Zimmermann S, Egeter O, Hausmann S, Lipford GB, Röcken M, Wagner H, Heeg K: CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J Immunol* 160: 3627–3630, 1998
17. Krieg AM: CpG DNA: a pathogenic factor in systemic lupus erythematosus? *J Clin Immunol* 15: 284–292, 1995
18. Miyata M, Kobayashi H, Sasajima T, Sato Y, Kasukawa R: Unmethylated oligo-DNA containing CpG motifs aggravates collagen-induced arthritis in mice. *Arthritis Rheum* 43: 2578–2582, 2000
19. Vinuesa CG, Goodnow CC:Immunology: DNA drives autoimmunity. *Nature* 416: 595–598, 2002
20. Gilkeson GS, Ruiz P, Pippen AM, Alexander AL, Lefkowith JB, Pisetsky DS: Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J Exp Med* 183: 1389–1397, 1996
21. Holdsworth SR, Kitching AR, Tipping PG: Th1 and Th2 T helper cell subsets affect patterns of injury and outcomes in glomerulonephritis. *Kidney Int* 55: 1198–1216, 1999
22. Nikolic-Paterson DJ, Atkins RC: The role of macrophages in glomerulonephritis. *Nephrol Dial Transplant* 16[Suppl 5]: 3–7, 2001
23. Deng GM, Nilsson IM, Verdrengh M, Collins LV, Tarkowski A: Intraarticularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat Med* 5: 702–705, 1999
24. Deng GM, Liu QZ, Tarkowski A: Intracisternally localized bacterial DNA containing CpG motifs induces meningitis. *J Immunol* 167: 4616–4626, 2001
25. Deng GM, Verdrengh M, Liu QZ, Tarkowski A: The major role of macrophages and their product tumor necrosis factor alpha in the induction of arthritis triggered by bacterial DNA containing CpG motifs. *Arthritis Rheum* 43: 2283–2289, 2000
26. Anders HJ, Vielhauer V, Cohen CD, Kretzler M, Segerer S, Luckow B, Weller L, Gröne HJ, Schlöndorff D: Chemokine and chemokine receptor expression during initiation and resolution of immune complex glomerulonephritis. *J Am Soc Nephrol* 12: 919–931, 2001
27. Austin HA, Muenz LR, Joyce KM, Antonovych TT, Balow EJ: Diffuse proliferative lupus nephritis: Identification of specific pathologic features affecting renal outcome. *Kidney Int* 25: 689–695, 1994
28. Satriano JA, Banas B, Luckow B, Nelson J, Schlöndorff D: Regulation of RANTES and ICAM-1 expression in murine mesangial cells. *J Am Soc Nephrol* 8: 596–603, 1997
29. Krieg AM: The role of CpG motifs in innate immunity. *Curr Opin Immunol* 12: 35–43, 2000
30. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the Lps gene product. *J Immunol* 162: 3749–3752, 1999
31. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11: 443–451, 1999
32. Leadbetter EA., Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A: Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607, 2002
33. Tsunoda I, Tolley ND, Theil DJ, Whitton JL, Kobayashi H, Fujinami RS: Exacerbation of viral and autoimmune animal models for multiple sclerosis by bacterial DNA. *Brain Pathol* 9:481–493, 1999
34. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM: Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 162: 6284–6293, 1999
35. Kitching AR, Tipping PG, Holdsworth SR IL-12 directs severe renal injury, crescent formation and Th1 responses in murine glomerulonephritis. *Eur J Immunol* 29: 1–10, 1999
36. Huang FP, Feng GJ, Lindop G, Stott DI, Liew FY: The role of interleukin 12 and nitric oxide in the development of spontaneous autoimmune disease in MRL/MP-lpr/lpr mice. *J Exp Med* 183: 1447–1459, 1996
37. Deng GM, Tarkowski A: Synovial cytokine mRNA expression during arthritis triggered by CpG motifs of bacterial DNA. *Arthritis Res* 3: 48–53, 2001
38. Takeshita S, Takeshita F, Haddad DE, Ishii KJ, Klinman DM: CpG oligodeoxynucleotides induce murine macrophages to up-regulate chemokine mRNA expression. *Cell Immunol* 206: 101–106, 2000
39. Mack M, Cihak J, Simonis C, Luckow B, Proudfoot AE, Plachy J, Brühl H, Frink M, Anders HJ, Vielhauer V, Pfirstinger J, Stangassinger M, Schlöndorff D: Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J Immunol* 166: 4697–4704, 2001