American Journal of Pathology, Vol. 162, No. 4, April 2003

Mononuclear Cell-Infiltrate Inhibition by Blocking Macrophage-Derived Chemokine Results in Attenuation of Developing Crescentic Glomerulonephritis

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Glomerular monocyte/macrophage (Mo/Mϕ) infiltrates play a role in many forms of glomerulonephritis (GN), and the intensity of Mo/Mϕ trafficking correlates with the loss of renal function and histological damage. We analyzed the functional role of macrophage-derived chemokine (MDC), a potent mononuclear cell chemoattractant, during the progression of anti-glomerular basement membrane (GBM) antibody (Ab) GN, a model of crescentic GN in the WKY rat, and whether the effects of MDC were dependent on its receptor CCR4. MDC mRNA and protein expression were markedly induced in nephritic glomeruli throughout the disease. Blocking the function of MDC did not affect the developing of the disease from days 2 to 7, but it dramatically blocked Mo/Mϕ infiltration in the glomeruli, prevented crescent formation, and reversed renal function impairment during days 7 to 14 of the anti-GBM GN. In this study, we also found that MDC activity on Mo/Mϕ in this GN was at least partly dependent on a new variant of CCR4. These results suggest that MDC is critically involved in the development of anti-GBM GN from acute glomerular injury to irreversible tissue damage. In addition, an antagonist to MDC may represent a prime drug target for therapeutic application to intervene in the progression of anti-GBM GN and in other Mo/Mϕ-dominant GN. ***(Am J Pathol 2003, 162:1061–1073)***

Leukocyte migration into glomeruli is a typical feature of glomerulonephritis (GN), and leukocytes are key mediators of kidney injury. Crescentic GN is a rapidly progressive glomerular disease that is usually associated with a poor prognosis. Although the pathogenesis of crescentic GN remains to be fully defined and likely involves several coordinated events, it is suggested that Mo/Mϕ play an important role.[1] Depletion of these cells by macrophage-specific antibodies or by irradiation reduces glomerular injury and proteinuria in experimental models.[2,3] Moreover, Mo/Mϕ are constituents of the crescents and are present in progressive GN; they probably play a major part in the irreversible scarring that leads to end-stage renal failure.[4,5]

Chemokines comprise a family of small proteins that are important in activating and recruiting leukocytes to sites of inflammation. Depending on the relative position of the first two cysteines, chemokines are divided into CC, CXC, C, and CX_3C subfamilies.[6,7] The CC chemokines usually act on monocytes, T lymphocytes, eosinophils, basophils, or mast cells. The repertoire of known human CC chemokines consists of more than 20 different molecules that have 25 to 70% identity with each other, and redundant biological activities have been observed. The overlapping spectrum of target cells is explained by the use of shared G-protein-coupled seven transmembrane domain receptors.[8]

Several studies have implicated chemokines as important pathophysiological mediators in glomerular injury. In crescentic GN, CXC chemokine expression, including macrophage inflammatory protein-2 (MIP-2), cytokine-in-

Supported in part by National Institutes of Health Grants DK20043 (C.B.W.) and DK54674–02 (L.F.). This is publication No. 14386-IMM from the Department of Immunology, The Scripps Research Institute, La Jolla, CA. Drs. Xia and Chen were the recipients of fellowships from the National Kidney Foundation of Southern California.

Accepted for publication December 18, 2002.

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duced neutrophil chemoattractant (CINC), and interferon-inducible protein 10 kd (IP-10), paralleled neutrophil influx during the acute evolution of anti-glomerular basement membrane (GBM) antibody (Ab) GN.[9–11] On the other hand, increases of T-lymphocytes and Mo/Mϕ coincided with the expression of the CC chemokines, RANTES (regulated on activation in normal T cells expressed and secreted), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1α), and macrophage inflammatory protein-1β (MIP-2β) in experimental anti-GBM GN.[11–13] Recruitment of inflammatory cells during acute renal allograft rejection and chronic allograft nephropathy has been suggested to be mediated by MCP-1, MCP-4, RANTES, MIP-1α, and MIP-1β.[14–18] Mice developing lupus nephritis have increased expression of RANTES, MCP-1, IP-10, MIP-1β, and BLC.[19,20] Indirect evidence from studies on MCP-1 in the rat model of remnant kidney suggests a functional role of this chemokine in glomerulosclerosis.[21–23] Chemokine expression has been analyzed in human kidney biopsies by *in situ* hybridization and immunohistochemistry. MCP-1 was detected in IgA nephropathy, proliferative GN, lupus nephritis, Wegener's granulomatosis, and acute interstitial nephritis.[24–27] MIP-1α and MIP-1β are also found in crescentic GN, Wegener's granulomatosis, and lupus nephritis.[24] CX3CL1 was detected in vasculitic GN.[28] The expression of chemokine receptors such as CXCR1, CXCR3, and CXCR5 is up-regulated in podocytes in patients with membranous nephropathy.[29]

Macrophage-derived chemokine (MDC)/stimulated T-cell chemotactic protein (STPC-1, CCL22) is a newly identified CC chemokine expressed by dendritic cells, B and T cells, and Mϕ. MDC shares less than 35% identity with any of the known chemokines, and has a tissue expression distribution nearly identical to that of thymus- and activation-regulated chemokine (TARC); like TARC, it has been shown to bind to CC receptor 4 (CCR4).[30–33] MDC attracts monocytes, dendritic cells, activated lymphocytes, eosinophils, and activated NK cells, and it has been identified as a $CD8^+$ T cell-derived factor capable of inhibiting the replication of both non-syncytium-inducing and syncytium-inducing isolates of HIV-1.[30–32,34] The expression of this novel chemokine is restricted to immune cells.

In Wistar-Kyoto (WKY) rats, a small dose of anti-GBM Ab induces severe proliferative and necrotizing GN with crescent formation and early infiltration of $CD8^+$ cells, followed by influx of Mo/Mϕ into the glomeruli.[35,36] In this model of GN, enhanced gene expression of chemokines has been demonstrated; however, most of the studies have focused on the acute phase of the disease.[13,37] Furthermore, studies with neutralizing antibodies and blocking reagents targeted acute glomerular injury and were used at day l, or at the same time as anti-GBM Ab administration, before the development of the disease.

In this study, the expression of MDC was analyzed in anti-GBM GN in WKY rats. In addition, the functional role of this novel chemokine between 2 to 7 days and 7 to 14 days in this model was defined. The effect of MDC was dependent on a variant of its receptor, CCR4. Blockade of MDC (on days 2 to 9) in this experimental anti-GBM GN lessened crescentic GN and development of renal failure between days 7 to 14.

Materials and Methods

Molecular Cloning of Rat MDC and CCR4

A rat spleen cDNA library in λ ZAP II vector (Stratagene, La Jolla, CA) was screened by hybridization using a rat MDC cDNA probe, which was generated by RT-PCR using rat macrophage RNA as a template with the primers designed according to conserved regions between murine and human MDC cDNA (murine cDNA GenBank accession no. AA175762, human cDNA GenBank accession no. NM_002990). PCR amplification was carried out using a proofreading DNA polymerase (platinum Pfx DNA polymerase; Invitrogen, Carlsbad, CA). Hybridization was performed as previously described.[38] In brief, approximately 5×10^5 recombinant phages from the cDNA library were screened with the ^{32}P-labeled oligonucleotide probe. Hybridization to filters was carried out overnight at 50°C in Hybrisol I (ONCOR, Norcross, GA), with 200 μg/ml of heat-denatured, sheared salmon sperm DNA, 5 μg/ml *E. coli* DNA, and a concentration of 2×10^6 cpm/ml of the probe. Filters were washed once with low-stringency wash buffer containing 2X SSC, 0.1% SDS at 37°C for 30 minutes, three times at 55°C for 30 minutes, and two times at 65°C for 20 minutes with high-stringency wash buffer containing 0.2X SSC and 0.1% SDS. Phagemids carried with λZAP II recombinants were rescued with helper phage (Stratagene). The cDNAs were subcloned into pBluescript vector, and were then sequenced by an ABI 373 automated DNA sequencer using T_7 and T_3 as primers. Since the full-length cDNA could not be obtained from the library, Rapid Amplification of cDNA Ends (RACE) was used to obtain the 5′ end fragment of rat MDC. RACE was performed as previously described with few modifications.[39,40] In brief, total RNA from spleen was first reverse transcribed by RNase H^- reverse transcriptase after annealing with oligo (dT) using a gene-specific primer (GSP) designed from the cloned rat MDC sequence (from 258 bp to 273 bp). A SuperScript Preamplification system (Invitrogen) and a proofreading DNA polymerase (platinum Pfx DNA polymerase, Invitrogen) were used for RT-PCR. After purification of first-strand product TdT tailing of cDNA was performed using dCTP and TdT (Invitrogen) at 37°C for 10 minutes. Tailed cDNA was then amplified by PCR with a proofreading DNA polymerase (platinum Pfx DNA polymerase, Invitrogen) using 5-μl volumes of the tailing reaction and the Abridged Anchore primer (5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′, Invitrogen) and nested gene-specific primer (from 237 bp to 257 bp). A nested amplification was then performed to obtain enough specific products. Nested PCR was conducting using Abridged Universal Amplification Primer (5′-GGC CAC GCG TCG ACT AGT AC-3′, Invitrogen) and a second nested primer (from 215 bp to 235 bp). 5′RACE products were cloned into pSTBlue-1 vector (Novagen, Madison, WI) and sequenced using T_7 and SP_6 as prim-

ers. The DNA and conceptualized protein sequence were made available to GenBank (accession number AF432871). A rat CCR4 cDNA fragment generated by RT-PCR was used as a probe to screen a rat thymus cDNA library (Stratagene) as previously described for rat MDC. The 320-bp CCR4 probe was cloned with primers designed according to the mouse and human conserved sequences, from bp 319 to 339 for sense and from bp 619 to 639 for antisense of the mouse CCR4 clone. Rat CCR4 was also short 20 amino acids at the 5′ end. RACE was used to generate the full-length rat CCR4. RACE was performed as described above for rat MDC. The following primers from the cloned rat CCR4 sequence were designed, for the first-strand synthesis a GSP from 569 pb to 584 bp was used, a nested primer was designed from bp 523 to 543 bp, and the second nested primer from bp 480 to 500 bp. A truncated product, shorter than the expected 5′ end, was preferentially amplified by RACE, sequence of this cDNA showed that this pool contained a product from bp 285 to bp 500. To avoid this truncated form, a third nested primer closer to 5′ end of CCR4 was designed, from bp 188 to 208. RACE products were cloned into T vector (pSTBlue-1, Novagen) and sequenced using T_7 and SP_6 as primers.

Identification of Chemokine Receptors Expressed in Nephritic Glomeruli of WKY Rats by PCR with Degenerated Oligonucleotides

Total RNA was isolated from nephritic glomeruli of WKY rats by the single-step method.[41] Poly(A)$^+$ mRNA was subsequently isolated by oligo (dt)-cellulose chromatography. Single-stranded cDNA was prepared from the resultant RNA by reverse transcription PCR. One-tenth of the reaction mixture was then subjected to 30 cycles of PCR using 3 μmol/L of each degenerated oligonucleotide primer (for sense, 5′-ATS GAY MGS TAC CTS GCY ATY GT-3; for antisense 5′-TTR TAR GGV RKC CAR MAG AGV A-3′). Reaction products migrating at the predicted size (350 to 400 bp) were gel-purified, subcloned into pBluscript, and sequenced using T3 and T7 primers.

Expression of Rat MDC and CCR4, Purification of Recombinant Proteins, and Generation of Antibodies

A PCR fragment encoding the mature form of MDC and N-terminal 48 amino acids of CCR4 were expressed as a His-tagged form and purified by Ni-NTA affinity column. A polyclonal antiserum was raised by immunizing a rabbit with recombinant MDC or CCR4 following previously described procedures.[42] The full-length rat MDC and CCR4 were subcloned into the mammalian cell expression vector pCDM8 (Invitrogen) and transfected into subconfluent 293T cells by electroporation. After 48 hours of incubation, the supernatant or the cell pellet were collected, electrophoresed in a 4 to 12% Bis-Tris Gel with 2-(N-morpholino) ethane sulfonic acid (MES) as a running buffer, transferred to nitrocellulose membrane, and blotted with various antisera.[43]

Deglycosylation of CCR4 Protein

Forty micrograms of cell lysate from 293T-transfectants with full-length CCR4 were denatured by heating to 95°C for 10 minutes after addition of SDS to 0.5% and β-mercaptoethanol to 1% (v/v). After addition of NP-40 detergent to 1% and addition of 2000 units PNGase F (New England Biolabs, Beverly, MA), the mixture was incubated at 37°C for 60 minutes, 12 hours, and 24 hours. The deglycosylation reaction was stopped by heating the samples at 100°C for 10 minutes. Sample control was treated as before but PNGaseF was substituted by reaction buffer (0.5 mol/L sodium phosphate, pH 7.5). Proteins were electrophoresed in a 4 to 12% Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane for Western blot.

Chemotaxis Analysis of Rat Monocytes and CCR4 293T Transfected Cells

Rat monocytes were isolated from PBMC using histopaque gradients (Sigma Chemical Co., St. Louis, MO). Migration was evaluated using a chemotaxis microchamber technique as previously described.[44] Twenty-five μl of increasing concentrations of 293T-derived MDC or control medium were placed in the lower wells of chemotaxis chamber (Neuro Probe, Gaithersburg, MD) and separated from 50 μl (1×10^6/ml) of cell suspension in the top of the wells by a 5-μm polycarbonate filter (Neuro Probe). Following incubation at 37°C for 2 hours, filters were removed and migrated cells on the undersurface were fixed with methanol and stained with Diff-Quik (American Scientific Products, McGraw Park, IL). Results are expressed as the mean number of migrated cells and are representative of $n = 3$ experiments performed in duplicate. Glomerular lysate from day 11 of WKY rats with anti-GBM GN was also used to study the chemotactic activity on monocytes as previously described. To demonstrate the specificity of MDC chemotactic activity, anti-MDC, anti-MCP-1, anti-RANTES, and anti-fractalkine Abs, generated in our laboratory, were pre-incubated with glomerular lysate before monocyte chemotaxis analysis. The effect of anti-MDC Ab in recombinant MDC-induced monocyte chemotaxis was used as a control.

Preparation of Nephrotoxic Serum

Nephrotoxic serum (NTS) was prepared by the method of Krakower and Greenspon with some modifications.[44] In brief, NZW rabbits were immunized with lysate of WKY rat glomeruli, isolated by differential sieving. The rabbit antiserum was heat-decomplemented and absorbed with rat blood cells and serum proteins. The specificity was confirmed *in vitro* by indirect immunofluorescence using fluorescein isothiocyanate conjugated (FITC) anti-rabbit IgG on frozen sections of normal WKY rat kidney. Sharp

linear immunofluorescence was observed in the GBM and the tubular basement membranes (data not shown).

Induction, Blocking Experiments, and Analysis of Crescentic Glomerulonephritis

All animal studies followed approved protocols conforming with USDA policies and the NIH Guide to the Care and Use of Laboratory Animals. At day 0, male WKY rats (Charles River Laboratories, Wilmington, MA), weighing 200 to 220 grams, received one intravenous injection of anti-GBM Ab at a dose of 2.5 μl/100 $\times$ *g* body weight. At various intervals from day 3 to 4 weeks, groups of three rats were euthanized to collect kidney tissue. Four groups of six rats each were given either 0.5 ml anti-MDC Ab or normal rabbit serum (NRS) intravenously for a period of 7 days, starting from day 2 after administration of NTS. Control and anti-MDC groups ($n = 6$) were then euthanized on days 7 and 14 to collect blood, and kidneys were removed for morphological and immunohistological analyses, as described below. Urine protein excretion was measured on timed 24-hour specimens collected at intervals from days 3 to 14, from individual rats in metabolic cages, and were assayed by the sulfosalicylic method. Urine and blood creatinine was determined using a creatinine diagnostic kit (Sigma).

Measurement of mRNA Expression by RNase Protection Assay (RPA)

For antisense riboprobe synthesis, the rat MDC (1–279, GenBank accession number AF432871) and its receptor CCR4 were used for *in vitro* transcription. A 350-bp CCR4 fragment (359–709, GenBank accession number AF432872) and a correspondent homology fragment (97–447, GenBank accession number AF432873) of a clone identified in the glomeruli of WKY rats by degenerated oligonucleotides RT-PCR were used for riboprobes. Total RNA was isolated from glomeruli using a single-step method.[41] Three μg of total RNA from each sample were used for RNase protection assay, following a previously described protocol.[45,46]

Immunoprecipitation and Western Blot Analysis

The protein levels of MDC in rat glomeruli and in supernatant transfectant cells were analyzed by Western blot analysis.[47] Isolated glomeruli from rats were homogenized in PBS with protease inhibitors. After centrifugation, the supernatants were collected and enriched by immunoprecipitation as previously described.[47] One-hundred μg of protein from each sample was immunoprecipitated with anti-MDC Ab using protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The immunoprecipitated samples and 5 μl of supernatant were electrophoresed in a NuPAGE gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane. The protein blot was first probed with biotinylated anti-MDC Ab and then with HRP-avidin D-conjugated second Ab (Vector Laboratories, Burlingame, CA), and developed with a SuperSignal kit (Pierce, Rockford, IL).

Morphological Analysis and Immunohistochemical Phenotyping and Quantitaion of Leukocytes

Kidney tissue samples were fixed in methanol-Carnoy fixative solution, embedded in paraffin, sectioned at 2 to 3 μm and stained with periodic acid-Schiff (PAS) reagent. Sections were examined for glomerular hypercellularity and necrosis, and formation of glomerular crescents. The number of crescentic glomeruli per 100 glomeruli of each rat was calculated and expressed as a percentage. For staining of $CD8^+$ and $ED1^+$ infiltrates, 5-μm paraffin sections of methanol-Carnoy fixed tissue were de-waxed and microwave-heated in 10 nmol/L sodium citrate (pH 6), as previously described.[44] The slides were reacted with mAb CD8a against rat CD8 (PharMingen, San Diego, CA)

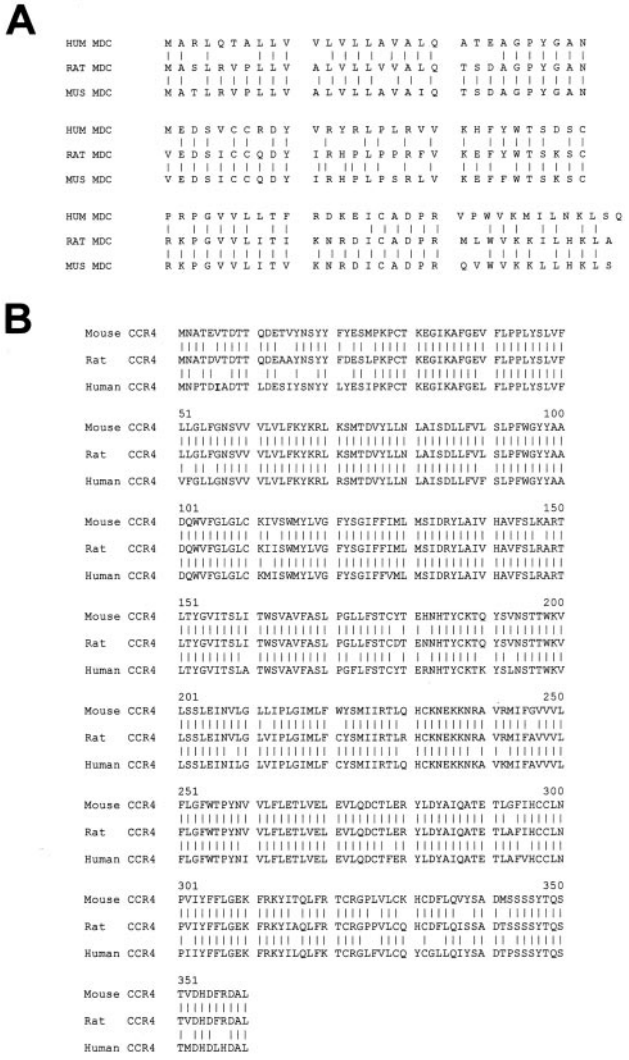

Figure 1. Alignment of amino acid sequence of MDC and CCR4. **A:** Alignment of amino acid sequence of human, rat, and mouse MDC. **B:** CCR4. Residues of identity are linked with **bars**. These sequence data are available from GenBank under accession nos. AF432871 and AF432872.

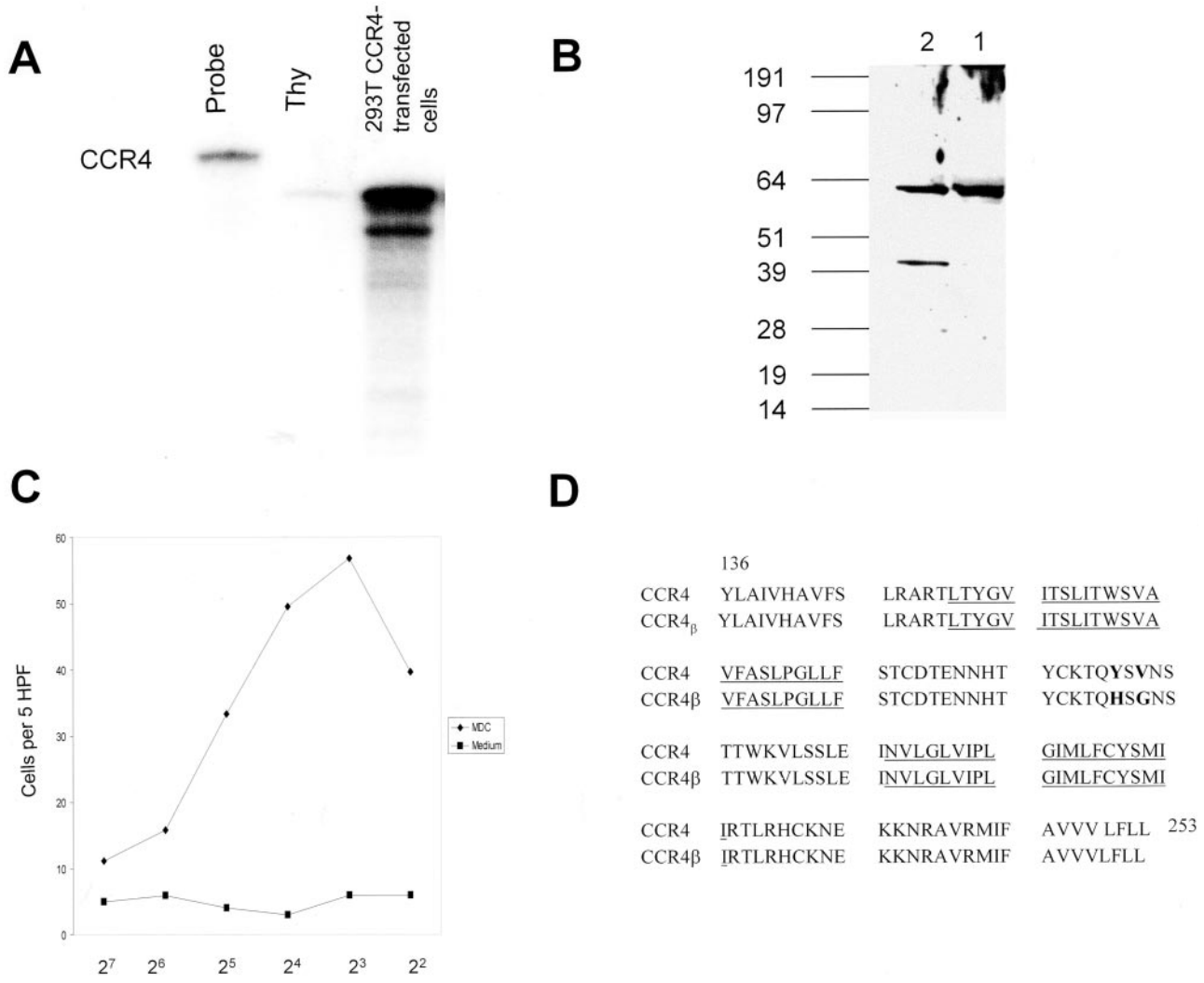

Figure 2. Expression of rat CCR4 in 293T cells and sequence of CCR4 and a variant of CCR4 (CCR4β). **A:** mRNA expression of CCR4. One μg of RNA from 293T cells expressing CCR4 was analyzed by RPA. Thymus was used as a positive control. Probe contains polylinker regions and is longer then the protected band. **B:** The cell pellet from 293T CCR4-transfected cells was collected for Western blot. Blot was probed with anti-rat CCR4 antibody. **Lane 1:** Denatured rat CCR4 demonstrating appearance of ~62kDa band. **Lane 2:** Deglycosylation of rat CCR4 by incubation with PNGase F (24 hours) resulted in the presence of a band of ~40 kd that correspond to the expected MW of rat CCR4. **C:** Chemotaxis assay. CCR4 293T transfected cells migrated with a bell-shaped dose-response curve in response to conditioned medium from MDC transfectants in comparison with medium alone as a negative control. **D:** Alignment of amino acid sequence of rat CCR4 and a new form of CCR4 (CCR4$_\beta$) is shown. A new variant of CCR4 was derived from nephritic glomeruli of WKY rats by PCR using degenerated oligonucleotides. The new variant contained two nucleotide substitutions that resulted in amino acid modification. Amino acid sequence variations are blackened. The underlined amino acids denote transmembrane regions of the molecules.

or mAb ED-1 against rat macrophages (Chemicon, Temecula, CA), and goat anti-mouse second antibody. Antibody binding was detected by an alkaline phosphatase antialkaline phosphatase kit and developed with a New Fuchsin substrate (DAKO Corp., Carpinteria, CA). Positively stained cells per 100 glomeruli of each rat were counted and expressed per glomerular section.

In Situ *Hybridization and Immunohistochemistry*

In situ hybridization was carried out using paraffin-embedded kidney sections. MDC riboprobe was synthesized with incorporation of ^{35}S-UTP as previously described.[10] Following *in situ* hybridization and before dehydration, the slides were incubated for 5 minutes in Tris-buffered saline. Endogenous peroxidase, avidin, and biotin were inactivated, and then sections were incubated with mAb ED-1 or mAb CD8a and with peroxidase-conjugated goat anti-mouse IgG, followed by mouse PAP. Sections were developed with amino-ethylcarbazole. After dehydration, emulsion-dipped slides were exposed for 3 weeks at 4°C then, developed, fixed, and counterstained with hematoxylin.[48,49]

Results

Cloning of Rat MDC and CCR4

Five positive clones, each 2.0 kb in size, were isolated for MDC. Sequence analysis revealed that these five clones were identical and all were partial cDNA clones contain-

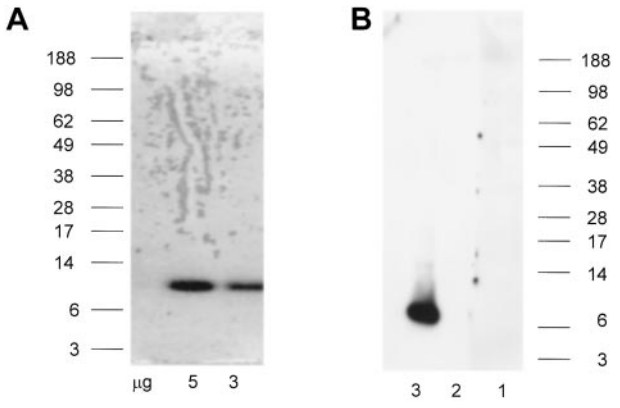

Figure 3. Expression of mature rat MDC and specificity of anti-MDC Ab. **A:** Expression of purified MDC after being subjected to Nu-PAGE gel electrophoresis and stained with Coomassie brilliant blue. **B:** Western blot of supernatants from 293T cells transfected with sense or anti-sense MDC in pCDM8. **Lane 1:** Pre-immune serum showed no reactivity with supernantant from 293T cells transfected with sense MDC. **Lane 2:** No reaction was seen with supernatant of 293T transfected with anti-sense MDC when reacted with anti-MDC. **Lane 3:** Anti-MDC reacted with the supernatant from the sense MDC transfected 293T cells used in **lane 1**.

ing only the 3′ end terminal coding sequence and 3′ untranslated region. A similar difficulty was encountered when cloning rat CCR4. Six positive clones were obtained. Sequence analysis revealed that the longest clone was short the N-terminal sequence encoding for 20 amino acids. To construct the full-length cDNA of MDC, RACE was used to clone the 5′ end as described in Materials and Methods. A RACE PCR product of around 280 bp was obtained (expected size 235 bp + 5′-UTR). The DNA sequence of this clone contained a translation initiation site (bp 32), and was 95% identical to mouse MDC in the coding region. The MDC was proved to be a non-error and fully functional form, since the riboprobe generated from the coding region of this clone was fully protected by MDC mRNA during RPA and the supernatant of the MDC cDNA transfectants induced monocyte chemotaxis. The sequence of the full-length cDNA of MDC contained an open reading frame encoding a 24 amino acid-long leader peptide and 68 amino acid-long mature protein with a predicted molecular mass of 7.8 kd. Rat MDC shares 86.9% and 67.4% identity at the amino acid level with mouse and human MDC, respectively (Figure 1A). The 5′ RACE product for CCR4 was also obtained with about 320 bp (expected size 208 bp + 5′ UTR) when rat thymus RNA was used as a template. This fragment included a translation initiation site (bp 86) after the polydG. The deduced amino acid sequence of full-length rat CCR4 was highly similar to mouse and human CCR4 (94.4% and 87.8%, respectively) (Figure 1B). A riboprobe generated from this clone (from bp 359 to bp 709) was fully protected when thymus sample, used as a control, and extracts from 293T expressing CCR4 were analyzed by RPA (Figure 2A). Western blot analysis of CCR4 protein from 293T CCR4-transfected cells detected a species of ~62 kd. Treatment of the protein with PNGaseF for 24 hours resulted in a significant increase in mobility and a shift from 62 kd to ~40 kd, similar to the expected molecular mass of CCR4. However, the 62-kd CCR4 was not completely deglycosylated as seen in Figure 2B. We next examined the ability of MDC to induce migration of CCR4 transfected 293T cells. As shown in Figure 2C, 293T cells expressing CCR4 migrated toward the increasing concentrations of MDC, whereas, transfected 293T cells failed to migrate when treated with medium alone. These chemotaxis results indicate that the cloned rat CCR4 encodes a functional chemokine receptor (Figure 2C).

Degenerated oligonucleotide primers based on the conserved regions of chemoattractant receptors were used in RT-PCR on glomeruli of WKY rats with CGN. Unexpectedly, 50% of the sequences analyzed encoded the CCR4 chemokine receptor. Alignment of the deduced amino acid sequence from one of these clones with our cloned rat CCR4 receptor (GenBank accession number AF432873) showed that these two receptors shared 98.3% identity (over 118 amino acids) (Figure 2D). The riboprobe transcribed from this fragment was fully protected in an RPA, indicating that the mismatch of nucleotides was not generated by PCR. In this form of CCR4 two novel nucleotide variants were identified, all of which were non-synonymous changes resulting in amino acid alterations. The two nucleotide variants were localized in the extracellular loop between transmembrane domains (TM) 4 and 5 (Figure 2D).

Expression of Rat MDC and Specificity of Anti-MDC Ab

The recombinant MDC was expressed as a fusion protein with a 6 X His tag at the amino termini. The protein had a higher molecular weight than expected due to the extra amino acids fused with MDC during expression (Figure 3A). The antiserum generated against MDC reacted monospecifically against the supernatant from 293T transfectants with rat MDC, but did not recognize any protein in supernatants from antisense transfectants as demonstrated by Western blot (Figure 3B).

In Vitro *Chemotactic Response of Rat Monocytes to MDC*

Simultaneous comparison of the chemotactic effect of glomerular lysate (day 11) from nephritic glomeruli of rats with CGN and supernatant from 293T MDC-transfected cells on rat monocytes, demonstrated comparable ability to induce monocyte chemotaxis by both samples (Figure 4A). When glomerular lysate-chemotaxis activity was blocked with anti-chemokine Abs, the chemoattractant activity of input monocytes was inhibited 57.4% by anti-MDC Ab, 25% by anti-MCP-1 Ab, and 0.6% by anti-RANTES Ab, but not by anti-fractalkine Ab (Figure 4B). To confirm the specificity of anti-MDC Ab, the chemotactic activity of MDC on rat monocytes was blocked by preincubation of supernatants from 293T-MDC-transfected cells with anti-MDC Ab. As a result, the chemotaxis response to MDC was efficiently inhibited by anti-MDC Ab (Figure 4C).

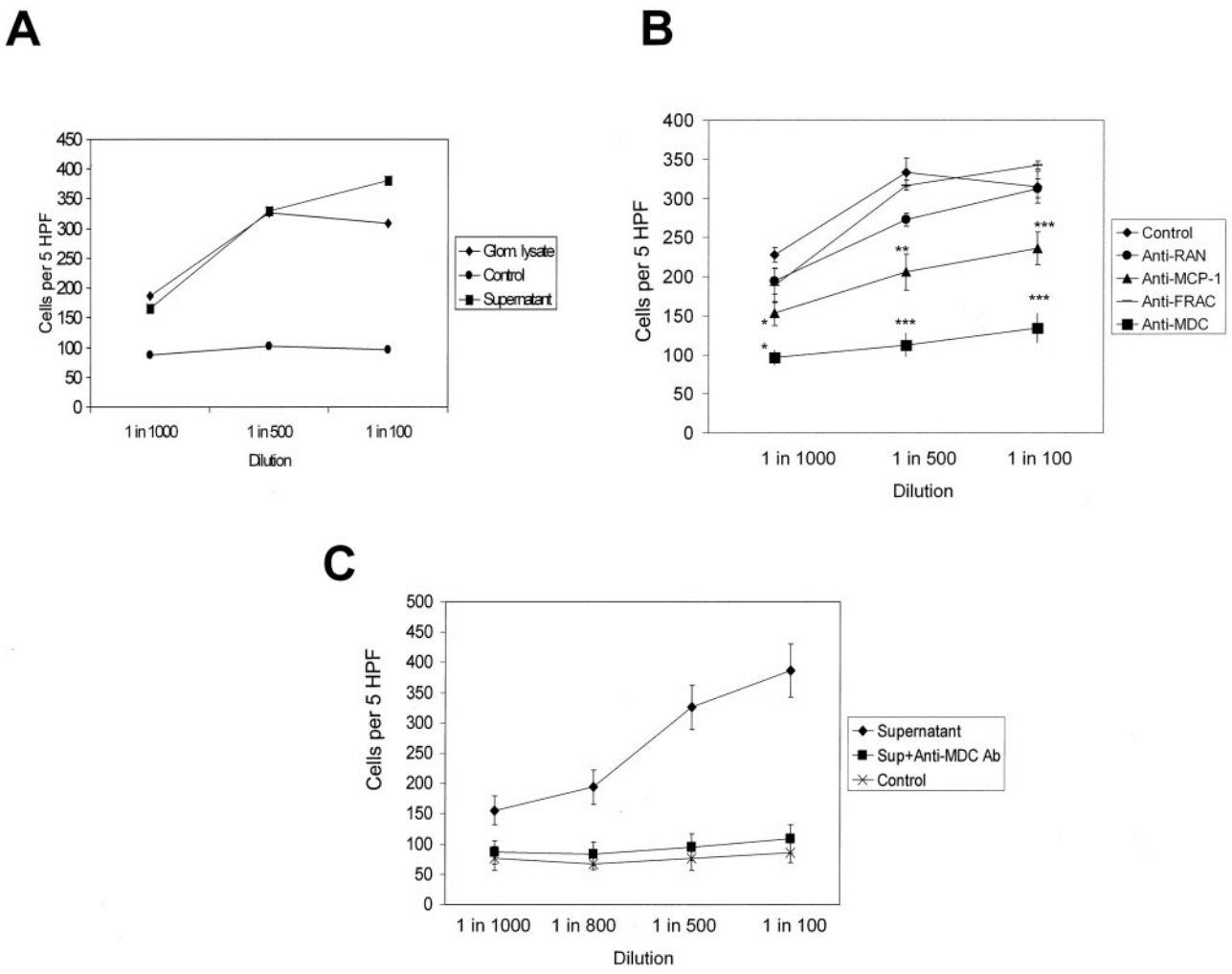

Figure 4. Chemotaxis of rat monocytes induced by MDC. **A:** Chemotactic response of rat monocytes to glomerular lysate (day 11) from rats with anti-GBM GN and supernatant from MDC-transfected cells. The chemotaxis response is expressed as number of migratory cells per five high-power fields (number/5X HPF). **B:** Chemotaxis inhibition of glomerular lysate by different chemokine antibodies. Abs were used at a concentration of 2 μg/ml. The assay was done in duplicate, and the number of migrating cells in five high-power fields was counted for each well. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ *t*-test. **C:** Anti-MDC Ab inhibition of chemotatic activity of supernatant from MDC-transfected cells. Supernatants (Sup) were pre-incubated with anti-MDC Ab at a concentration of 2 μg/ml before rat monocyte chemotaxis analysis.

Rat MDC Expression in Glomeruli During the Course of Crescentic GN

To determine the role of MDC in the recruitment of leukocytes to sites of inflammation *in vivo*, its expression was determined in glomeruli throughout the development of anti-GBM GN (day 3 to day 30). Normal glomeruli had very little mRNA and protein expression of MDC, but from day 3 onwards NTS treatment induced strong expression of this chemokine. Densitometric analysis demonstrated that the level of glomerular mRNA for MDC was more than 100 times higher in rats with anti-GBM GN on day 3 than in control animals, peaked on day 7, stayed at similar levels until day 15, and started to subside by day 21 (Figure 5A). Western blot analysis of rat MDC protein confirmed that MDC protein expression correlated with their mRNA level in the glomeruli (Figure 6). We further examined the expression of CCR4, which has been reported as a receptor for MDC. In contrast with the increased expression of MDC, the expression of CCR4 mRNA was not increased in nephritic glomeruli (Figure 5B). However, the expression of MDC correlated with the time-course expression of the variant CCR4 mRNA (Figure 5C).

Morphological Analysis and Characterization of Inflammatory Infiltrates

To define the functional role of MDC in the development of crescentic GN, a blocking experiment with anti-MDC Ab was performed. Once anti-GBM GN was established in WKY rats (day 2), a neutralization Ab against MDC or NRS was administrated daily from day 2 to day 9, and on days 7 and l4 rats were sacrificed. On day 7, a slight but significant reduction of crescents (7.8%) was observed in the rats treated with anti-MDC Ab (Figure 7). However, other difference in histological appearance of kidneys was not observed between the rats treated with NRS and anti-MDC Ab (data not shown). At this point, severe glo-

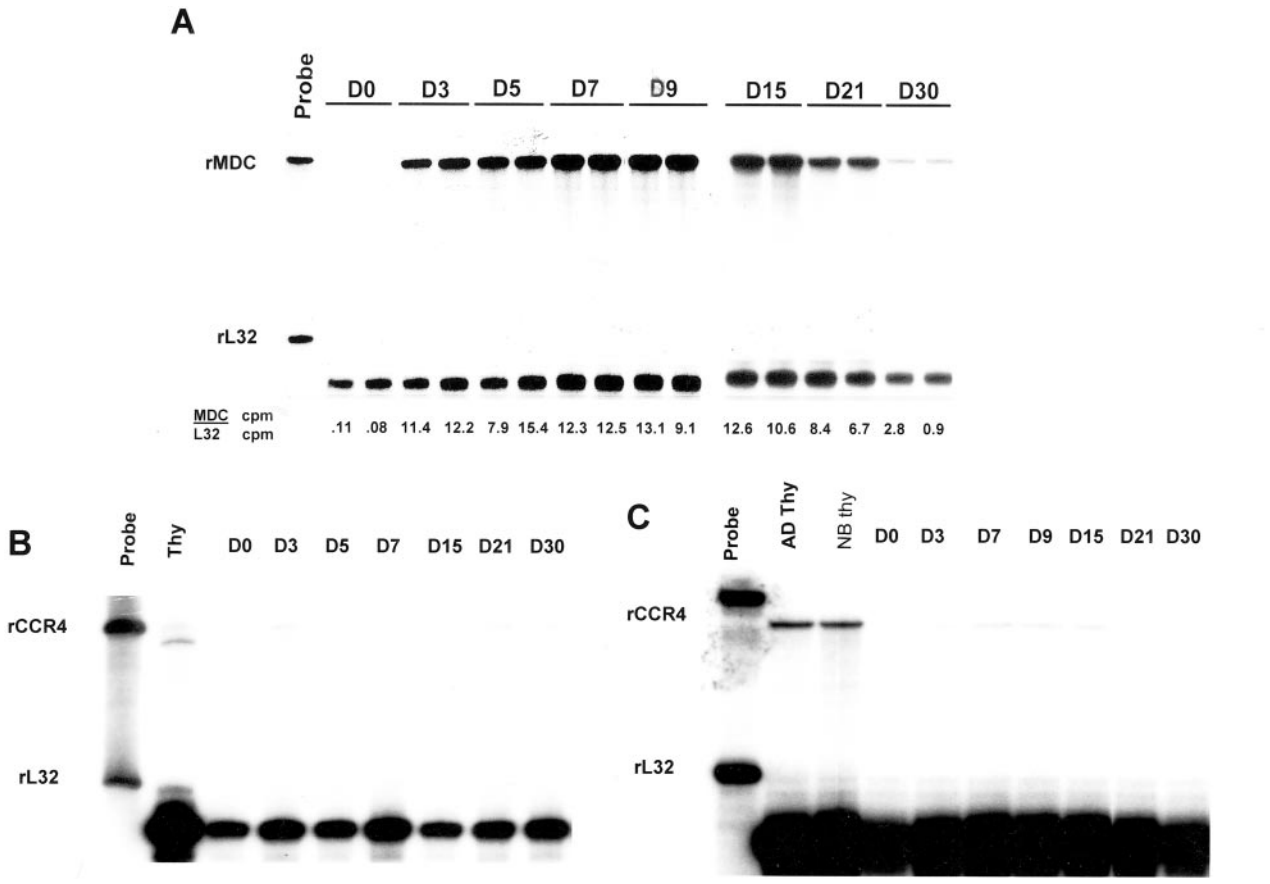

Figure 5. Analysis of MDC and CCR4 expression in the glomeruli of WKY rats with anti-GBM GN. Glomeruli from WKY rats were isolated at various time points after induction of GN. **A:** RPA analysis of MDC mRNA expression. Each **lane** represents a single rat sample. The probe contains polylinker regions and is longer than the protected band. The expression levels of MDC mRNA were quantitated on the AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, CA). The final values are expressed relative to the mRNA levels of the housekeeping gene L-32. **B:** CCR4 mRNA expression. **C:** mRNA expression of a variant of CCR4. (AD thy, thymus from adult rat; NB thy, thymus from newborn rat). Each **lane** represents one rat sample.

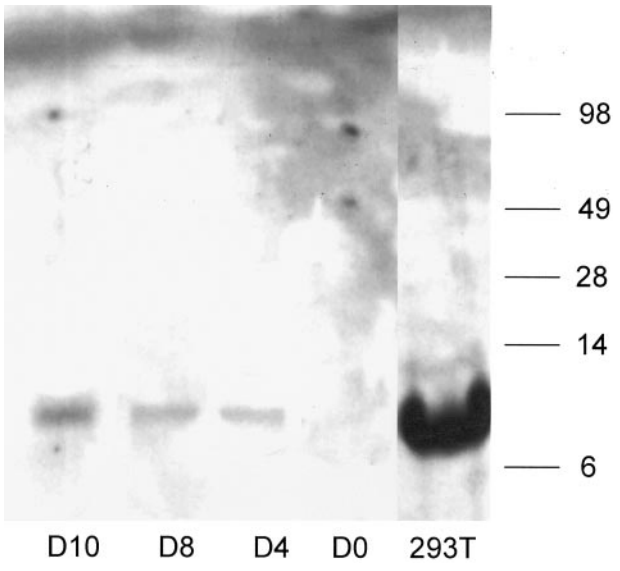

Figure 6. Analysis of MDC protein expression in the glomeruli of anti-GBM GN in WKY rats. One hundred μg of protein from each sample was used. Supernatant from 293T MDC-transfected cells was used as a positive control. Results shown are representative of three independent experiments.

merular hypercellularity was observed in both groups. Quantitative study indicated a prominent glomerular and periglomerular accumulation of $CD8^+$ cells (data not shown) and $ED1^+$ Mo/Mϕ (Figure 7). On day 14, the glomerular hypercellularity, focal areas of necrosis, and crescentic formation were markedly reduced in the anti-MDC Ab-treatment group (Figures 7 and 8). Immunohistochemical phenotyping revealed that anti-MDC Ab resulted in a striking decrease (44.6%) in intraglomerular $ED1^+$ infiltrates (Figures 7 and 8). The blocking effect was specific, since immunogen-adsorbed Ab did not affect the infiltrate (data not shown). As expected, $CD8^+$ cells were not observed in either NRS or anti-MDC Ab-treatment groups because this model of GN is characterized by an early infiltration of $CD8^+$ cells (maximum increase on day 3). *In situ* hybridization demonstrated that at day 7 to 15 of anti-GBM GN, MDC was confined to Mo/Mϕ by immunohistochemistry (Figure 9).

Analysis of Renal Function

All rats that received NTS and NRS showed significant proteinuria on day 5 and throughout the course of the study. There was no difference in 24-hour urinary protein after anti-MDC Ab treatment in the acute phase of anti-

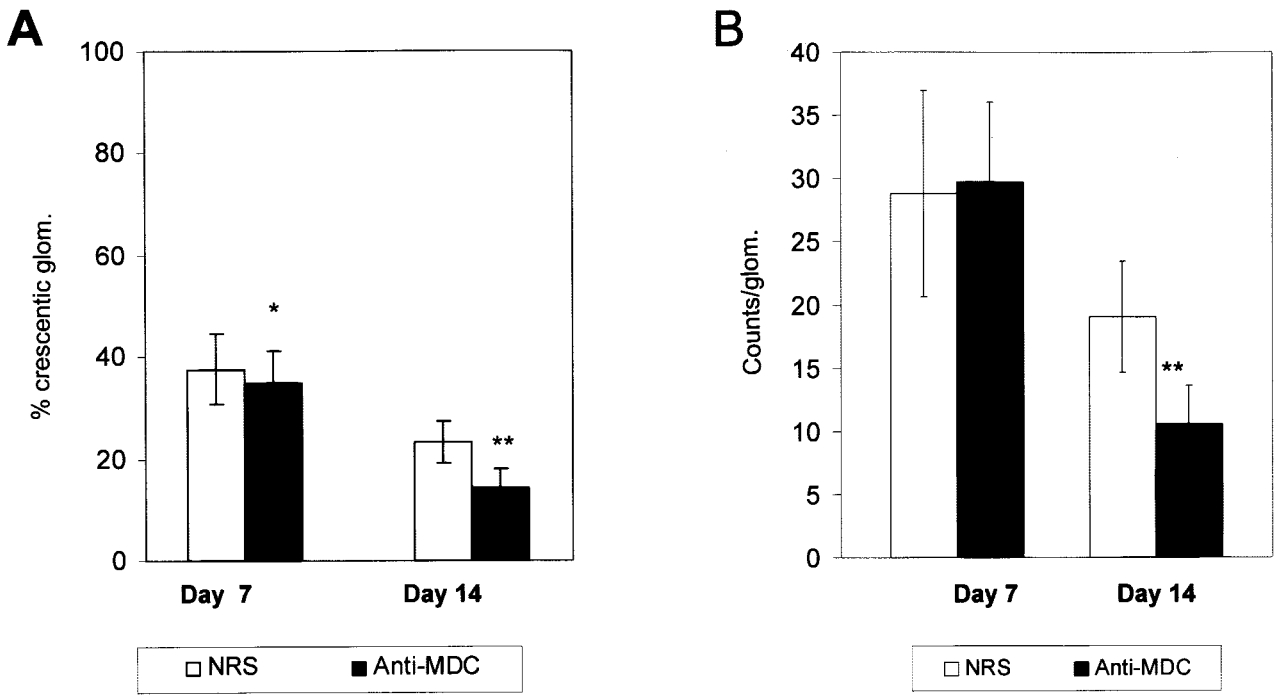

Figure 7. Quantitation of glomerular crescent formation (**A**) and ED1$^+$ cell infiltration (**B**). **A**, glomeruli containing crescents were calculated in PAS-stained sections. One hundred glomeruli per section were counted. **B**, the numbers of ED1$^+$ cells were enumerated in immunohistochemically stained sections. Each data point represents sections sampled from six rats ($n = 6$) and is expressed as mean ± SD. *, $P < 0.044$; **, $P < 0.0001$

GBM GN. However, anti-MDC Ab treatment prevented the increase in proteinuria in the progressive phase of anti-GBM GN, with a significant reduction of 52.2% at day 9 (32.76 ± 1.18 *vs.* 16.93 ± 6.18 mg/d, $n = 6$ per group). The reduction in protein excretion on days 11 and 13 was more dramatic with 80.2% (54.75 ± 18.03 *vs.* 10.56 ± 4.7 mg/d, $n = 6$ per group) and 75% (36.4 ± 17.53 *vs.* 9.83 ± 5.05 mg/d, $n = 6$ per group), respectively (Figure 10A). On day 7, slightly higher levels of SCr (0.44 ± 0.01 *vs.* 0.5 ± 0.01 mg/dl, $n = 6$ per group) and lower levels of CCr (1.13 ± 0.16 *vs.* 0.91 ± 0.32 ml/min, $n = 6$ per group) were observed in rats treated with anti-MDC Ab (Figure 10, B and C). At day 14, serum creatinine levels were significantly lower (1.04 ± 0.13 *vs.* 0.38 ± 0.045 mg/dl, $n = 6$ per group) and the creatinine clearance significantly higher (0.36 ± 0.12 *vs.* 1.73 ± 0.29 ml/min, $n = 6$ per group) after administration of anti-MDC Ab (Figure 10, B and C).

Discussion

Anti-GBM GN in WKY rats is a severe form of crescentic GN, characterized by glomerular infiltration of CD8$^+$ and Mo/Mϕ. The peak influx of Mo/Mϕ occurs later (days 6 and 11) than that of CD8$^+$ cells (day 3).[35] In this study we investigated the role of MDC, a novel C-C chemokine for monocytes, activated lymphocytes, dendritic cells, and NK cells in the development of this model of GN. We first examined MDC expression in the glomeruli during the evolution of the lesion and then established its functional role with blocking Ab. We found that a significant induction of MDC was correlated with the influx of CD8$^+$ lymphocytes and mononuclear phagocytes. To investigate the role of MDC during the induction and development of the disease, a blocking experiment with anti-MDC Ab was performed during anti-GBM GN.

Although MDC was expressed in 3 to 7 days of anti-GBM GN, anti-MDC Ab treatment started at day 2 slightly decrease crescent formation (7.8%), however, did not prevent proteinuria or produce significant inhibition of Mo/Mϕ infiltration at day 7 of the GN. These results indicate that other chemokines that are also expressed at this time, such as MCP-1, RANTES, MIP-1 β, and fractalkine, may play a predominant role.[44] Moreover, in a similar model of GN in rats, blocking the function of MCP-1 reduced leukocyte infiltration and proteinuria.[13] Similar results were found in mice, blocking the function of MCP-1 or RANTES.[12] Blocking MIP-1α and MIP-1β in anti-GBM GN in rats resulted in a 60% reduction of the acute phase proteinuria without reduction in leukocyte influx.[9]

Anti-MDC Ab administration resulted in significant attenuation of Mo/Mϕ infiltration, concomitant with a marked reduction in crescent formation at day 14 of anti-GBM GN. As a result of the attenuation of inflammatory lesions in the kidney, renal function was largely maintained in rats with crescentic GN treated with anti-MDC Ab.

Chemotaxis assay of the glomerular lysate of WKY rats in the progressive phase of anti-GBM GN (day 11) demonstrated that MDC was responsible for 57.4%, MCP-1 for 25%, and RANTES for 0.6% of Mo/Mϕ infiltration at this time of the disease. These data suggest that MDC is the main chemokine determining the infiltration of Mo/Mϕ at this phase of disease in this model. The role of MCP-1

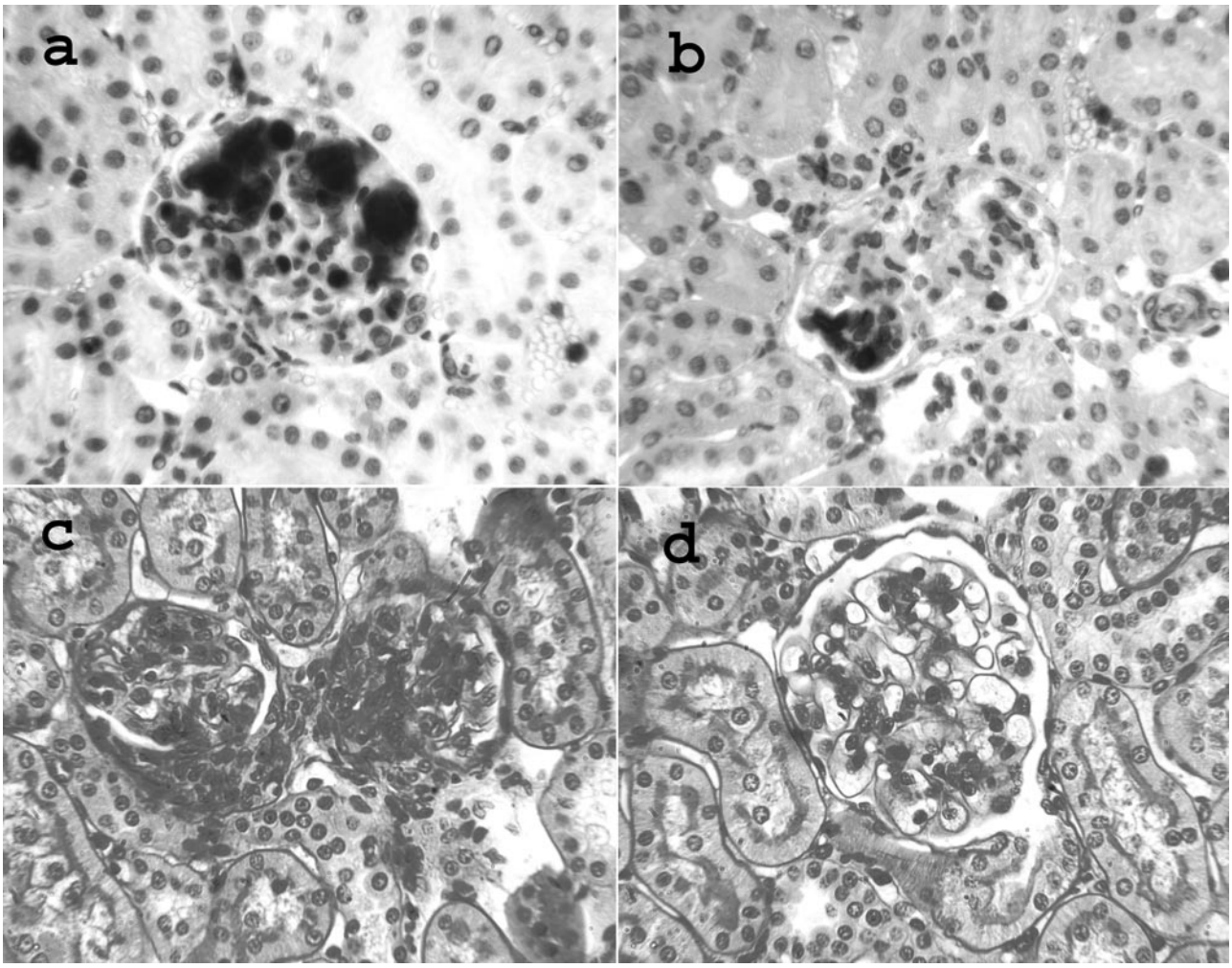

Figure 8. Photomicrographs (original magnification, ×400) of glomeruli from WKY rats with anti-GBM GN that were treated with normal rabbit serum (NRS) or with anti-MDC Ab. Immunohistochemistry stains for ED1$^+$ Mo/Mϕ (**A** and **B**) of kidney sections from rats treated with NRS (**A**) or anti-MDC Ab (**B**). Sections were sampled on day 14 after anti-GBM Ab injection. PAS (**C–D**) staining of kidney sections of NRS (**C**) or anti-MDC Ab (**D**) treated rats.

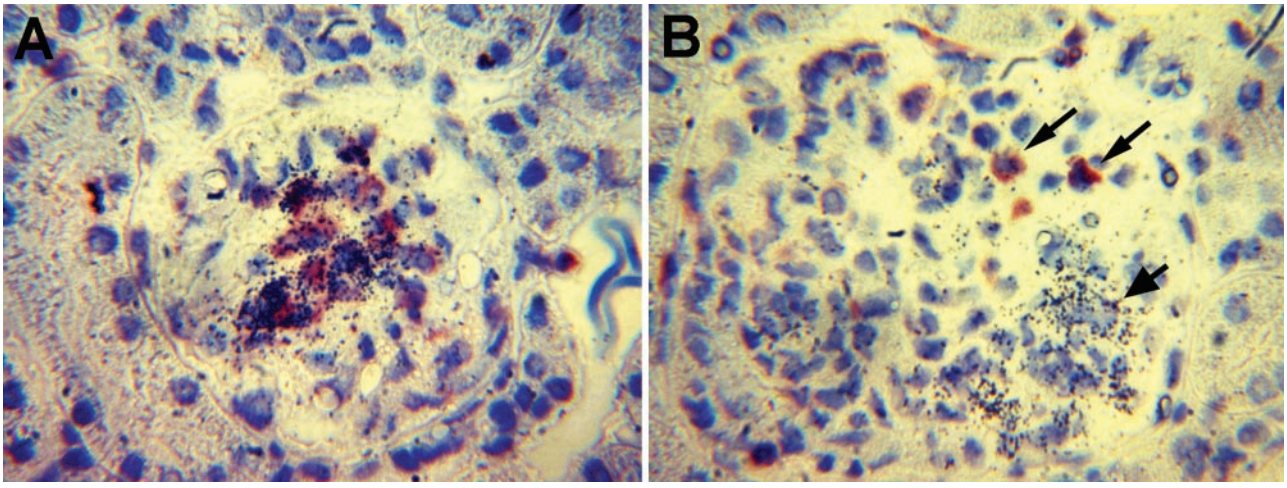

Figure 9. *In situ* hybridization and immunohistochemistry of the glomeruli from rats with anti-GBM GN. Simultaneous detection of MDC mRNA (black grains) in ED1$^+$ Mo/Mϕ (brown) by *in situ* hybridization and immunohistochemistry (**A**), but not in CD8$^+$ cells (brown) (**B**). Sections were sampled on day 7 after anti-GBM Ab injection.

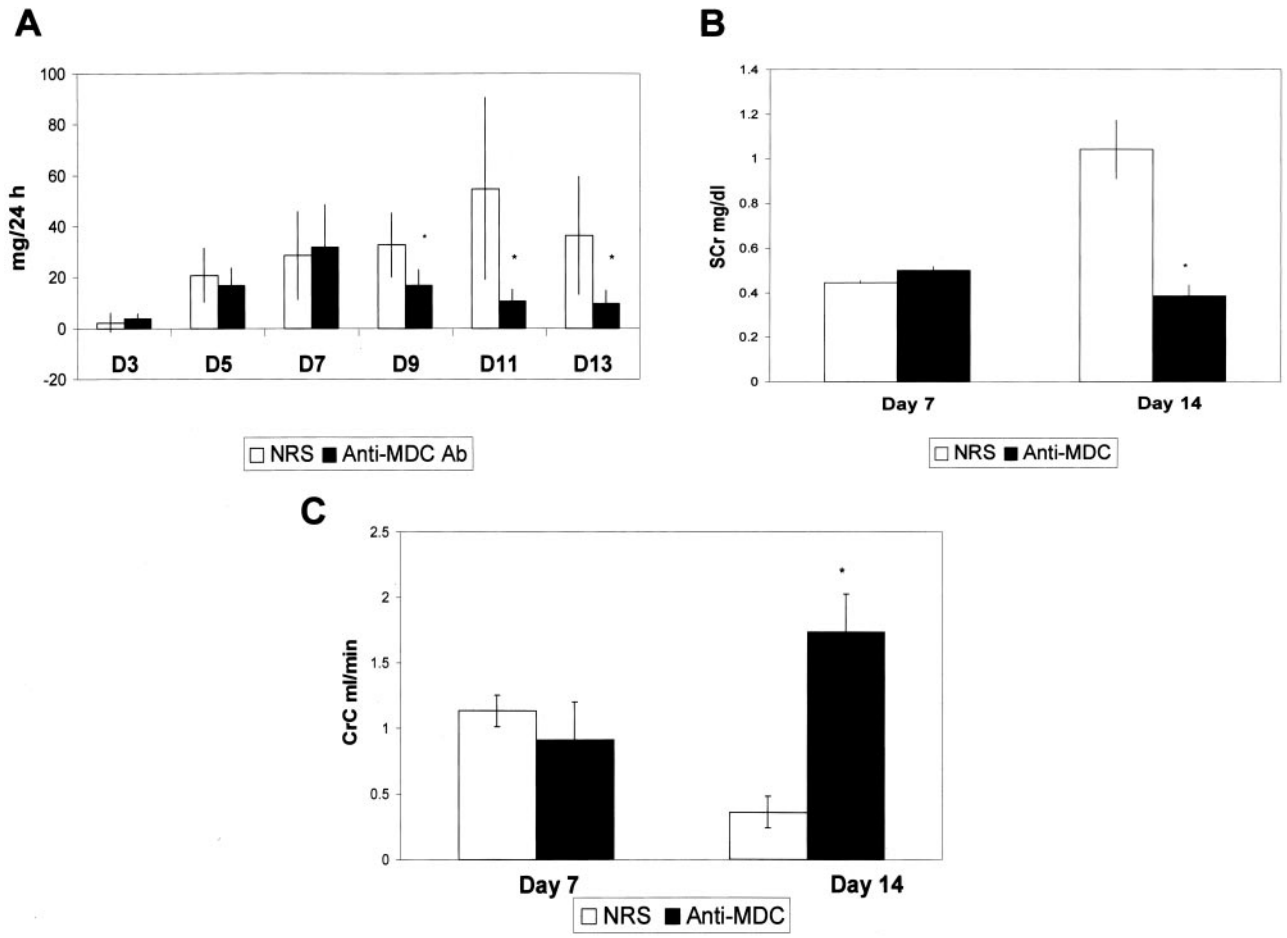

Figure 10. Proteinuria (**A**) in WKY rats with anti-GBM GN treated with NRS or anti-MDC Ab. Serum creatinine levels (**B**) and creatinine clearance (**C**) in rats that were treated with NRS or anti-MDC Ab. Results were sampled from six rats per group ($n = 6$) and expressed as mean ± SD. *, $P < 0.01$.

and possibly other chemokines may explain the incomplete inhibition of Mo/Mϕ infiltration by MDC neutralization at this phase, and emphasize the role of biological redundancy in the chemokine system. Accordingly, in anti-GBM GN in WKY rats, neutralization of MCP-1 reduced glomerulosclerosis and improved renal function in the later phase of the disease.[37] In a similar model of GN in mice, neutralization of antibodies to MCP-1 resulted in a decrease in the deposition of collagen.[12] Nonetheless, Fujinaka et al[13] found a significant suppression of glomerular infiltration and proteinuria by blocking MCP-1 at the early phase of the disease but not at day 8. This discrepancy could be because of a difference in the rout and the time of administration of the blocking Ab.

The ability to attenuate histopathological damage and to reverse renal function impairment by targeting only MDC is impressive, considering that other chemokines are also produced during the progression of anti-GBM GN. The MDC-mediated suppression of disease progression was associated with the reduction of glomerular Mo/Mϕ accumulation in anti-MDC Ab-treated rats compared to NRS-treated animals. Glomerular Mo/Mϕ are associated with glomerular hypercellularity, crescent formation, glomerulosclerosis, tubular atrophy, and interstitial fibrosis.[4,5] The reduced glomerular Mo/Mϕ infiltrates imply a subsequent decrease in Mo/Mϕ-derived mediators such as cytokines, growth factors, nitric oxide, and reactive oxygen intermediates, that are involved in glomerular injury.[50,51] The inhibition of Mo/Mϕ infiltration appears to be an important measure in preventing the progression of acute glomerular injury to irreversible tissue damage. Platelet activation by MDC has been demonstrated;[52] whether the attenuation of anti-GBM GN blocking the function of MDC is also mediated by affecting platelet function needs to be documented.

The positive surface charge has been proposed to be critical for chemokine heparin binding. Stromal cell-derived factor-1 is a highly basic chemokine with a predicted pI of 10.66 (Genetics Computer Group program) that binds heparin with higher affinity than MCP-1 (pI 9.4). Determination of MDC pI showed that MDC is also highly basic protein (pI 9.93). This characteristic could allow a strong binding of MDC to glycosaminoglycanes and in this form retain it at sites of production, and may explain its marked expression throughout the anti-GBM GN and contribute to maintain glomerular inflammation.

MDC is expressed in immune cells; accordingly, we did not find expression of MDC by resident renal cells by *in situ* hybridization. Most of the dendritic cells of the kidney reside within the glomerular mesangium. The early

expression on MDC probably proceeds from these mesangial dendritic cells; MDC may attract $DC8^+$ and subsequent influx of Mo/Mϕ, with a further accumulation of MDC and amplification of the inflammatory response. By *in situ* hybridization and immunohistochemistry, we found that Mo/Mϕ were the main source for MDC in nephritic glomeruli at days 7 to 14 of the disease. Interestingly, not all Mo/Mϕ expressed MDC; this may reflect selective expression of MDC by specific Mϕ phenotypes, as demonstrated by the absence of MDC expression in lung and liver, where Mϕ are also found. In addition, some Mo/Mϕ from nephritic glomeruli might not fully differentiate into mature phenotypes, in which MDC is highly expressed.[30]

Although MDC binds CCR4, in this study we could not find a correlation between the increased expression of MDC and CCR4, because RPA failed to reveal induction of CCR4 mRNA in the glomeruli of rats with anti-GBM GN. MDC may recognize another, yet unidentified receptor. Dendritic cells and monocytes, cell types responsive to MDC, do not express CCR4; TARC binds CCR4, however, TARC does not block MDC-mediated Ca^{2+} flux in Th2 lymphocytes.[53] In addition, CCR4 is expressed selectively on Th2 cells; however, Th1 and Th2 cells are attracted by MDC.[31] Furthermore, it has been reported that truncated MDC (3–69) has reduced chemotactic activity for lymphocytes, but not for monocytes.[54] Degenerated oligonucleotide-based RT-PCR was used to identify candidate MDC receptors in nephritic glomeruli. The results showed a variant of CCR4 that paralleled its ligand mRNA expression. mRNA of this CCR4 variant was also found expressed in thymus.

This new form of CCR4 presented two variations in nucleotide sequences at positions that are highly conserved in CC chemokine receptors. Both variations were identified in the extracellular loop between TMD 4 and 5. Variations in the extracellular domains in another CC receptor, CCR5, have been related to modifications in specific ligand-induced functions. Therefore, further studies will be required to obtain full characterization of this new and possible other forms of CCR4. This is an important issue because of the central role MDC plays in inflammatory disease models, such as this model of GN, as well as allergic inflammation and its potent anti-HIV effect.[34,55]

In conclusion, this study provides evidence that MDC plays a critical role in the influx of Mo/Mϕ to glomeruli at day 7 to 14 of renal injury in experimental anti-GBM GN, and in the development of the ensuing irreversible tissue damage and progression to renal failure. Moreover, this study also indicates that an antagonist to MDC might be a drug target for therapeutic application to intervene in the progression anti-GBM GN and in other Mo/Mϕ-dominant GN.

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