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Inhibition of CXCL16 Attenuates Inflammatory and Progressive Phases of Anti-Glomerular Basement Membrane Antibody-Associated Glomerulonephritis

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Chemokines recruit and activate leukocytes during inflammation. CXCL16 is a recently discovered chemokine that is expressed as a transmembrane protein that is cleaved to form the active, soluble chemokine. We analyzed the role of CXCL16 in the development of inflammation and in the progression of the anti-glomerular basement membrane (GBM) antibody-induced experimental glomerulonephritis in Wistar-Kyoto rats. CXCL16 was expressed in glomerular endothelial cells and mediated adhesion of macrophages expressing CXCL16 and its cognate receptor, CXCR6. Glomerular infiltrates displayed a strong migratory response to soluble CXCL16. Soluble CXCL16 and its receptor CXCR6 were induced in nephritic glomeruli throughout the disease, and CXCL16 expression correlated with the up-regulation of ADAM10, suggesting that this disintegrin and metalloproteinase mediates the chemokine activity of CXCL16. Blocking CXCL16 in the acute inflammatory phase or progressive phase of established glomerulonephritis significantly attenuated monocyte/macrophage infiltration and glomerular injury; proteinuria also improved. We conclude that CXCL16/CXCR6 plays a critical role in stimulating leukocyte influx, which causes glomerular damage during anti-GBM glomerulonephritis. Blocking CXCL16 actions limits the progression of anti-GBM glomerulonephritis even when the disease is established. (*Am J Pathol* 2007, 170:1485–1496; DOI: 10.2353/ajpath.2007.060065)

The migration of leukocytes from blood vessels into tissue is central to the process of inflammation in the development of many kidney diseases.^{1–5} The recruitment of leukocytes involves a cascade of cellular events including mechanisms of cell chemotaxis and adhesion. Chemokines are cell-selective molecules that are present in gradients that provide directional cues for invading leukocytes.^{6–8} To affect the circulating leukocytes, tissue-derived secreted chemokines are immobilized on the luminal membrane of endothelial cells by interaction with glycosaminoglycans or by internalization and transcytosis at the luminal surface.^{9,10}

Induction of chemokines and infiltration of chemokine receptor-bearing cells occurs in animal models of renal diseases and in human kidney diseases or renal allograft rejection. For example, the expression of CXCL1/MIP-2 and CXCL10/IP-10 correlates with neutrophil influx in anti-glomerular basement membrane (GBM) antibody (Ab) glomerulonephritis (GN) in Lewis rats.^{11,12} In Wistar-Kyoto (WKY) rats with anti-GBM GN, CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL22/MDC, and CX₃CL1/fractalkine are expressed.^{1,3,13} In mice with accelerated nephrotoxic serum nephritis, CCL5/RANTES and CCL2/MCP-1 increase in relationship to infiltration of T lymphocytes and macrophages.⁴ CCL2/MCP-1 has also been detected in IgA nephropathy, proliferative GN, lupus nephritis, Wegener's granulomatosis, and acute interstitial nephritis.^{14–16} CCL3/MIP-1 α and CCL4/MIP-1 β are expressed in crescentic GN, Wegener's granulomatosis, and lupus nephritis.¹⁶ This is relevant because there is evidence that down-regulation of chemokine signals can suppress leukocyte influx into the glomeruli or interstitium of the kidney.¹⁷

CXCL16 is a chemokine with a transmembrane domain. Its expression on the surface of antigen-presenting cells, dendritic cells, CD19⁺ B cells, and CD14⁺ mono-

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cytes/macrophages is up-regulated by inflammatory mediators and lipopolysaccharide.^{18,19} CXCL16 is not secreted and is expressed on T cells, aortic smooth muscle cells, and umbilical endothelial cells, whereas its receptor, CXCR6 (STRL33/BONZO/TYMSTR), is expressed on T cells from inflamed tissues, such as rheumatoid joints and inflamed livers, and on natural killer T cells, aortic smooth muscle cells, astrocytes, epithelial cells, and stromal cells.^{20–28} CXCL16 functions both as a transmembrane adhesion molecule and as a membrane metalloprotease-cleaved soluble chemoattractant for CXCR6-bearing cells. ADAM10, a disintegrin and metalloproteinase, has been identified in the ectodomain shedding of this novel chemokine.^{29,30}

CXCL16 in macrophages was identified as a scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein.^{31,32} Although CXCL16 has been suggested to participate in endocarditis, experimental hepatitis, rectal cancer, and experimental autoimmune encephalomyelitis, its role in inflammatory kidney diseases is unknown.^{31–36}

We have investigated the role of this novel adhesive chemokine in progressive anti-GBM GN in WKY rats. This is a severe and progressive, cell-mediated model of inflammation in which non-helper type lymphocytes take part in the pathogenesis of GN.^{37–39}

CXCL16 was expressed in glomerular endothelial cells (GECs) and significantly attracted leukocytes to infiltrate the glomeruli. When we blocked the function of CXCL16 during either the acute inflammatory phase or established glomerulonephritis, there was significant attenuation of invading monocytes/macrophages and glomerular injury. These results suggest that this membrane-associated chemokine is involved in the development of inflammation and disease progression.

Materials and Methods

Molecular Cloning of Rat CXCL16 and CXCR6

Primers for rat CXCL16 and CXCR6 cDNA were generated from conserved sequences of the mouse and human genes. For reverse transcription-polymerase chain reaction (PCR), 5 μ g of total RNA from thymus or bone marrow-derived macrophages were first reverse-transcribed by Moloney murine leukemia virus reverse transcriptase after annealing with oligo(dT). The reverse transcripts were used as a template for PCR amplification performed using a proofreading DNA polymerase (platinum Pfx DNA polymerase; Invitrogen, Carlsbad, CA) as described.⁴⁰ Products were cloned into pBluescript vector and then sequenced by an ABI 373 automated DNA sequencer using T₇ and T₃ as primers (Applied Biosystems, Foster City, CA).

Expression of Rat CXCL16, Generation of Green Fluorescent Protein (GFP)-Fused CXCL16, and Confocal Microscopy

cDNA encoding the open-reading frame of CXCL16 was cloned into mammalian expression vector pcDNA3. Hu-

man embryonic kidney (HEK) 293 cells were transfected by electroporation with 10 μ g of plasmid DNA. To determine the localization of CXCL16 in living cells, GFP-fused CXCL16 was generated. The plasmid pGFPemd (BD Biosciences Clontech, Palo Alto, CA) was used as a template for PCR to incorporate appropriate restriction enzyme sites and link GFP at the CXCL16 3' end. HEK293 cells were transfected by electroporation with 10 μ g of pcDNA3GFP-fused CXCL16 or pcDNA3GFP plasmid alone. After 24 hours, cells were washed two times with phosphate-buffered saline and then fixed for 30 minutes on ice in 4% formaldehyde in PEM buffer (80 nmol/L potassium PIPES, pH 6.8, 5 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, pH 7.0, and 2 mmol/L MgCl₂). After fixation, cells were washed with PEM buffer and incubated 5 minutes with 1 mg/ml sodium borohydride to quench autofluorescence and then washed twice with PEM, counterstained with 4,6-diamidino-2-phenylindole, and viewed by fluorescence confocal microscopy (excitation 4,6-diamidino-2-phenylindole 360/40, GFP 490/20; emission 4,6-diamidino-2-phenylindole 475/50, GFP 528/30).

Cell Culture

T lymphocytes were purified from spleen with anti-T-cell (OX52) MicroBeads (Miltenyi Biotec Inc., Auburn, CA) and activated with 400 U/ml interleukin (IL)-2 as described.⁴¹ Peritoneal macrophages from normal rats were collected by washing the peritoneal cavity twice with 50 ml of phosphate-buffered saline. Alveolar macrophages were isolated as described.⁴² Primary rat GECs were a gift of Dr. Stephen Adler (New York Medical College, Valhalla, NY). Their endothelial origin was demonstrated by virtue of morphology, uptake of diI(1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine)-acetylated low-density lipoprotein, and angiotensin-converting enzyme activity.^{43,44} Cells were used at passages 8 to 15.

Measurement of mRNA expression by RNase protection assay (RPA). For antisense riboprobe synthesis, the rat CXCL16 (274 nucleotides) and its receptor CXCR6 (393 nucleotides) were used for *in vitro* transcription. L-32 (92 nucleotides) was generated by PCR using a cDNA template and was used as a housekeeping gene. Rat ADAM10 cDNA (GenBank accession no. Z48444) was used for preparation of riboprobe. All plasmids were linearized with EcoR1 digestion and transcribed with T7 RNA polymerase using [α -³²P]UTP. Total RNA was isolated using a single-step method.⁴⁵ The RPA was performed following a previously described protocol using a kit (Torrey Pines Biologicals, Houston, TX).^{3,46,47} In brief, 3 μ g of glomerular total RNA was hybridized with 10⁵ counts of each [³²P]UTP-labeled rat antisense probe. Unhybridized RNA was digested with RNase at 30°C for 30 minutes. The RNase was denatured with stop buffer, and then the samples were electrophoresed on 6% polyacrylamide gel. Phosphorimage quantification was performed using the PhosphorImager SI scanning instrument and ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).^{13,48,49}

Generation of Anti-Serum Against CXCL16

A PCR-amplified cDNA fragment encoding the chemokine domain and part of the mucin domain of rat CXCL16 (amino acids 26 to 159) protein was cloned in frame into the *E. coli* expression vector pETM1. The plasmid was transformed into *E. coli* host strain BL21(DE3) for the expression and purification of His-tagged recombinant protein. The purified recombinant protein was injected into a rabbit to raise antiserum following previously described procedures.⁵⁰ To characterize the antiserum generated against CXCL16 [anti-CXCL16 rabbit serum (RS)], total protein extract from spleen was used in Western blot analysis. Previously, it has been demonstrated that spleen constitutively expresses CXCL16.^{18,19} Supernatants and cell lysates from HEK293 cells that had been transfected with expression plasmid encoding full-length CXCL16 and cells lysate from GECs that constitutively express CXCL16 were also used to characterize anti-CXCL16 RS. To determine the neutralizing activity of the anti-CXCL16 RS, we performed chemotaxis assay using lymphocytes and peritoneal macrophages as a target cells. Recombinant CXCL16 was preincubated with CXCL16 RS 30 minutes at 37°C before the assay.

Adhesion Assay

Adhesion assay was performed as previously described.⁵¹ In brief, HEK293 cells were transfected by electroporation with pCDNA3CXCL16 and cultured on an eight-well slide chamber until confluence. The transfection efficiency was ~60%. After washing with phosphate-buffered saline, nonspecific binding sites were blocked with 1% bovine serum albumin in 20 mmol/L HEPES, pH 7.4. Peritoneal macrophages (1×10^5) were added to the wells and incubated at 25°C for 1 hour. After washing off nonadherent cells, remaining cells were fixed with 1% glutaraldehyde. To determine whether adhesion was mediated by CXCL16, transfected cells were preincubated with anti-CXCL16 RS before the adhesion assay. Next, we examined whether the adhesion was a function of the chemokine portion of the molecule by using recombinant soluble CXCL16. To investigate whether CXCL16-induced adhesion could be physiologically relevant, we induced GEC-CXCL16 with IL-1 β (10 ng/ml) for 4 hours before the addition of peritoneal macrophages. To determine whether macrophage adhesion was mediated by CXCL16, GECs were preincubated with anti-CXCL16 RS, and then macrophages were exposed to GECs. Assays were performed in triplicate, and the percentage of adherent cells was determined by counting cells in 10 $\times$ 400 fields per well.

Preparation and Chemotaxis Analysis of Inflammatory Leukocytes from Nephritic Glomeruli

Inflammatory leukocytes were isolated from the nephritic glomeruli of WKY rats 7 and 15 days after anti-GBM GN induction, following the method of Cook et al.⁵² The in-

flammatory cells were characterized by examination of stained cytopins; at day 7 cells were primarily monocytes/macrophages (75%), with some lymphocytes (25%). At day 15, most of the cells consisted of monocytes/macrophages (~85%). Cell viability was ~90% as determined by trypan blue. Migration of inflammatory leukocytes was evaluated using a chemotaxis microchamber technique as described.¹¹ Results were expressed as mean $\pm$ SD cell number per five high-power fields ($\times$ 400) and were a representative of $n = 3$ experiments performed in duplicate.

Immunoprecipitation and Western Blot Analysis

The protein levels of CXCL16 in rat glomeruli were analyzed by Western blot analysis.^{13,49} Isolated glomeruli from rats were homogenized in phosphate-buffered saline with protease inhibitors. After centrifugation, the supernatants were collected and enriched by immunoprecipitation. One hundred μ g of protein from each sample was immunoprecipitated with anti-CXCL16 Ab using protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The immunoprecipitated samples were electrophoresed on a NuPage gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane. The protein blot was first probed with biotinylated anti-CXCL16 Ab and then with HRP-avidin D-conjugated second Ab (Vector Laboratories, Burlingame, CA) and developed with a SuperSignal kit (Pierce, Rockford, IL). Blots were stripped and then reprobed for β -actin as a housekeeping gene.

Flow Sorting

Inflammatory leukocytes isolated at day 7 after the induction of anti-GBM GN were used to identify macrophages as previously described.³ In brief, cells were collected from 10 rats with anti-GBM GN and pooled to yield sufficient cell material for sorting ($\sim 1 \times 10^7$ /ml). Cells were reacted with fluorescein isothiocyanate-labeled anti-ED1 mAb or fluorescein isothiocyanate-labeled anti-CD45RA mAb OX-33 as a control. A high-speed sorter was prepared for aseptic sorting, and after appropriate gating and compensation setting, stained cells were gated according to their forward scatter area versus side scatter area characteristics to exclude lymphocytes and debris. A re-sort analysis of sorted cells was run to verify purity, which was found to be ~98%. Sorted cells were immediately spun down and resuspended in media to improve recovery (~70%). Cells were collected for RNA purification to perform reverse transcription-PCR to detect the presence of CXCL16 and CXCR6. PCR was performed for 30 cycles. The expected size of the PCR product was 437 bp for CXCL16 and 393 bp for CXCR6. In control PCR reactions, reverse-transcribed samples following RNase treatment was used.

In Situ Hybridization

In situ hybridization was performed to identify CXCL16 expression in anti-GBM GN. Immunohistochemistry was

not used because the expected cross-reactivity and high background with the use of the second antibody since both anti-GBM and anti-CXCL16 antibodies were produced in rabbits. A fragment of 274 bp (from nt 88 to nt 361 from rat CXCL16) with an *Eco*R1 site at 5' and a *Bam*H1 at the 3' end was subcloned into pGEM4Z (Promega, Madison, WI). The recombinant plasmid was linearized with *Eco*R1 or *Bam*H1 and transcribed with T7 or SP6 RNA polymerases to generate anti-sense and sense probes, respectively. *In situ* hybridization was performed using paraffin-embedded kidney sections as described previously.^{11,13} Rat CXCL16 riboprobe was synthesized with incorporation of ³⁵S-UTP. After autoradiography for 3 days, the slides were dipped in Kodak NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with water at 42°C. Exposure was done at 4°C in the dark for 4 weeks before development.

Induction, Blocking Experiment, and Analysis of Anti-GBM GN in WKY Rats

All animal studies followed approved protocols conforming with United States Department of Agriculture policies and the National Institutes of Health Guide to the Care and Use of Laboratory Animals. A rabbit antiserum to rat glomerular basement membrane was prepared as described.^{1,13} At day 0, male WKY rats (Harlan, Indianapolis, IN), weighing 200 to 220 g, received one intravenous injection of anti-GBM Ab at a dose of 25 μ l/100 g of body weight. Four groups of eight rats each were given either 100 μ l of anti-CXCL16 RS or normal rabbit serum (NRS) intraperitoneally for a period of 7 days, starting from day 1 after administration of anti-GBM Ab. Control and anti-CXCL16 RS groups ($n = 8$) were then euthanized on days 8 and 16. At day 8 there is significant glomerular accumulation of CD8⁺ and macrophages, and histological changes are well developed. At day 16, the stage of chronic inflammation, marked crescent formation, severe necrotizing lesion, and fibrotic changes are established.

To examine whether the antiserum to CXCL16 would affect the course of the progressive phase of established GN, we measured proteinuria in rats with anti-GBM GN. The rats were paired according to the degree of proteinuria, and one rat of each pair was treated with the anti-CXCL16 RS from days 7 to 11. Likewise, we paired rats with anti-GBM GN on day 10 and began treatment for 5 days. Urine protein of rats in individual metabolic cages was measured on timed 24-hour specimens from day 3 to 15 by the sulfosalicylic method. The rats were euthanized on days 12 and 16, respectively. Kidneys were removed for morphological and immunohistological analyses as described below.

Morphological Analysis and Immunohistochemical Phenotyping and Quantitation of Leukocytes

Kidney tissue samples were fixed in methanol-Carnoy fixative solution, embedded in paraffin, sectioned at 2

μ m, and stained with periodic acid-Schiff (PAS) reagent. For staining of CD8⁺ and ED1⁺ infiltrates, 5- μ m paraffin sections of methanol-Carnoy-fixed tissue were dewaxed and microwave-heated in 10 nmol/L sodium citrate, pH 6, as previously described.^{1,48} The slides were reacted with mAb CD8a against rat CD8 (BD Pharmingen, San Diego, CA) or mAb ED-1 against rat macrophages (Chemicon, Temecula, CA), followed by goat anti-mouse secondary Ab. Antibody binding was detected by an alkaline phosphatase antialkaline phosphatase kit.

Histological Evaluation

Renal tissue sections of each animal stained with hematoxylin & eosin (H&E), periodic acid-Schiff, and trichrome stains were examined without knowledge of the experimental condition. The following changes were recorded and quantified. 1) Glomerular hypercellularity was expressed as the mean score of 70 to 100 glomeruli, each of which was scored as 0 = normal, 1 = hypercellularity in <25% of glomerular capillary area, 2+ = 25 to 50%, 3+ = 50 to 70%, and 4+ = >75%. 2) Glomerular fibrinoid necrosis was expressed as the percentage of glomeruli showing this change. 3) Glomerular sclerosis was expressed as the percentage of glomeruli with sclerosis in >50% of glomerular capillary areas. 4) Crescentic formation was expressed as the percentage of glomeruli showing this change. 5) Chronic tubulointerstitial injury was scored as 0 = normal, 1 = <25% cortical area, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = >75%. There were no significant vascular changes for scoring.

Statistics

The unpaired *t*-test was used to determine differences between treatment groups in the *in vivo* studies. In the chemotaxis assay, analysis of variance and the Bonferroni multiple-comparison test was used. *P* values less than 0.05 were considered statistically significant.

Results

Cloning of Rat CXCL16 and CXCR6

The rat and mouse CXCL16 amino acid sequences were 77.2% homologous, and the mature chemokine domain was 87% homologous (Supplemental Figure 1, see <http://ajp.amjpathol.org>). The CXCL16 clone we isolated was a non-error and fully functional form. A riboprobe generated from the coding region was fully protected by CXCL16 mRNA during RPA. The supernatants from cells transfected with the cloned cDNA had CXCL16 as determined by Western blot, and it induced chemotaxis of CXCR6-bearing cells (Figure 1A). Comparison of rat and mouse CXCR6 amino acid sequence showed 88.3% homology.

Figure 1. Rat CXCL16 is present in both soluble and membrane-bound forms. **A:** Chemotaxis assay. The bell-shaped dose-response curve relationship of lymphocyte migration in response to conditioned medium from CXCL16 transfectants differed markedly from chemotaxis in response to media alone. To determine the distribution of CXCL16 in living cells, GFP-fused CXCL16 cDNA was cloned into mammalian expression vector pcDNA3 and transfected into HEK293 cells by electroporation. The cells were visualized by confocal fluorescent microscopy. **B:** HEK293 transfected with pcDNA3GFP-CXCL16 showed that CXCL16 is a membrane-anchored protein. **C:** HEK293 transfected with pcDNA3GFP alone demonstrated a predominantly cytoplasmic distribution of GFP.

Membrane CXCL16 Expression

Transfection of HEK293 cells with a plasmid encoding GFP-fused rat CXCL16 demonstrated that this chemokine is expressed as a cell surface ligand (Figure 1B). Control

Figure 3. Specificity of anti-CXCL16 Ab. **A:** Western blot of total protein extracted from spleen. **Lane 1:** Anti-CXCL16 RS reacted against spleen (spleen cells constitutively express CXCL16 protein). We found a band of ~60 kDa that corresponds to the full-length CXCL16.¹⁸ **Lane 2:** Preimmune serum did not react against spleen. **B:** The anti-CXCL16 RS reacted with proteins in the supernatants and cell lysates from HEK293 cells that had been transfected with a full-length CXCL16 in pcDNA3. The expected band of ~35 kDa was identified in the supernatant (**lane 1**). The cell lysate contained a 60-kDa form that corresponds to the full-length chemokine and a 34-kDa form (presumably a cleaved form or an intracellular precursor, **lane 2**). In lysates from untreated GECs, there was only the 34-kDa band (**lane 3**). The anti-CXCL16 RS neutralized chemotaxis of lymphocytes (**C**) or macrophages (**D**). CXCL16 was preincubated with different dilutions of anti-CXCL16 RS. The chemotactic response was inhibited by anti-CXCL16 RS but not by NRS. * $P < 0.001$ versus CXCL16 or NRS, ** $P < 0.05$ anti-CXCL16 RS 1:200 versus anti-CXCL16 RS 1:500.

cells transfected with plasmid encoding GFP alone showed a predominantly cytoplasmic distribution of GFP (Figure 1C).

Expression of CXCL16 and CXCR6 mRNA in Cultured Kidney Cells and Inflammatory Cells

Using RPA, we found constitutive expression of CXCL16 in GECs. Proinflammatory stimuli increased GEC-CXCL16 expression (Figure 2A), whereas IL-1 β induced CXCL16 expression in mesangial cells (Figure 2B). Peritoneal macrophages expressed both the ligand and its receptor, but alveolar macrophages expressed only CXCL16 without its receptor (Figure 2C).

Characterization of Anti-CXCL16 Antibody

Western blot analysis demonstrated that the antiserum generated against CXCL16 (but not the preimmune serum) reacted against spleen, which constitutively expresses CXCL16 (Figure 3A).^{18,19} The antiserum generated against rat CXCL16 reacted against a ~35-kDa protein in the supernatants of HEK293 cells that had been transfected with pcDNA3CXCL16, corresponding to the soluble form of this chemokine.¹⁸ The anti-serum also reacted with proteins of ~60 and ~34 kDa present in the

Figure 2. CXCL16 and CXCR6 mRNA expression in cultured kidney cells and inflammatory cells. **A–C:** RNase protection assay of CXCL16 and CXCR6 mRNA expression. **A:** Induction of CXCL16 in GECs stimulated with IL-1 β (10 ng/ml), tumor necrosis factor (TNF)- α (100 ng/ml), interferon (IFN)- γ (100 U/ml), or lipopolysaccharide (LPS; 1 μ g/ml) for 4 hours. CXCL16 mRNA was constitutively expressed and cytokines or LPS stimulated GEC-CXCL16 expression. **B:** CXCL16 mRNA was induced in mesangial cells (MC) stimulated by IL-1 β (50 ng/ml) for 4 hours. **C:** Peritoneal macrophages (PM ϕ) expressed both CXCL16 and CXCR6, but lung M ϕ only expressed CXCL16 mRNA. Rat ribosomal L32 gene was used as a loading control. The expression of CXCL16 and CXCR6 mRNAs are expressed relative to the mRNA of the housekeeping gene L32. Probes contain polylinker regions and, hence, are longer than the protected bands.

Figure 4. Membrane-anchored CXCL16 mediates cell adhesion. **A:** The adhesion of peritoneal macrophages to HEK293 cells transfected by electroporation with a mammalian expression plasmid encoding CXCL16 was evaluated. Mock transfected cells were used as a control. HEK293 cells expressing CXCL16 supported adhesion of macrophages and an excess of soluble recombinant CXCL16 (chemokine domain, 3 nmol/L) or anti-CXCL16 RS (1:200 dilution) reduced the adhesion of macrophages. **B:** GECs activated by IL-1 β (10 ng/ml) for 4 hours induced the adhesion of peritoneal macrophages. Pretreatment of GECs with anti-CXCL16 RS significantly attenuated CXCL16-induced macrophage adhesion. * P < 0.001 versus control, ** P < 0.001 versus HEK293-CXCL16 or IL-1 β -activated GECs.

cell lysates (Figure 3B). The 60-kD protein corresponds to the full-length transmembrane chemokine, whereas the ~34-kD protein is presumably a proteolytically cleaved product or possibly an intracellular precursor.¹⁸ Anti-CXCL16 RS also recognized a ~34-kD protein in quiescent GECs (Figure 3C).

We analyzed the ability of anti-CXCL16 RS to suppress the chemotactic activity of CXCL16 in cells expressing CXCR6. Dilutions of anti-CXCL16 RS efficiently inhibited chemotactic activity of both lymphocytes and peritoneal macrophages (Figure 3, C and D).

Figure 5. Chemotaxis to CXCL16 and analysis of CXCL16 and ADAM10 expression in the glomeruli of WKY rats with anti-GBM GN. **A:** Chemotaxis of inflammatory leukocytes from the glomeruli of WKY rats with anti-GBM GN. Infiltrating inflammatory cells at day 7 were primarily macrophages (75%) with some lymphocytes (25%) but at day 15 were mainly macrophages (~85%). Chemotaxis was expressed as number of migratory cells per five high-power fields. Media were used as a control for CXCL16. * P < 0.001 versus control, λP < 0.001 versus 10^{-7} M, and δP < 0.001 versus 10^{-8} M. **B:** RNase protection analysis of CXCL16 and CXCR6 mRNA expression. CXCL16 and CXCR6 were induced during anti-GBM GN (the additional bands reflect the excess of radiolabeled RNA probe). The expression of CXCL16 and CXCR6 mRNAs are expressed relative to the mRNA L-32 as a control. **C:** Western blot analysis of glomeruli CXCL16. During anti-GBM GN soluble CXCL16 protein (the 35-kD band) was induced in glomeruli. As a control, supernatants and cell lysates from HEK293 that had been transfected with pcDNA3CXCL16 were examined. β -Actin was used to evaluate loading. **D:** RNase protection analysis of ADAM10 mRNA. ADAM10 mRNA expression was induced during anti-GBM GN. The expression levels of ADAM10 mRNA are corrected for L-32 mRNA.

Figure 6. Identification of CXCL16 and CXCR6 mRNAs in macrophage infiltrates from nephritic glomeruli. **A:** CXCL16 was identified by reverse transcription-PCR in macrophages from nephritic glomeruli. Lanes: M, marker; 1, thymus; 2, macrophages from nephritic glomeruli; 3, negative control (RNA from macrophages isolated from nephritic glomeruli pretreated with RNase); and 4, bone marrow-derived macrophages (BMDM). **B:** Macrophages from nephritic glomeruli express CXCR6. Lanes: 1, macrophages; 2, negative control; 3, thymus; and 4, BMDM.

CXCL16 Mediates the Adhesion of CXCR6-Expressing Cells

We investigated whether membrane-bound CXCL16 mediates adhesion of monocytes/macrophages by transfecting HEK293 cells with pcDNA3CXCL16 and adding peritoneal macrophages. Transfected HEK293 cells yielded adhesion of peritoneal macrophages (Figure 4A). Preincubation of HEK293 cells with anti-CXCL16RS blocked the adhesion of macrophages, indicating that cell adhesion was indeed mediated by CXCL16 (Figure 4A). The addition of the soluble form of recombinant CXCL16 reduced the adhesion of macrophages, suggesting that the chemokine portion of the protein is required for adhesion (Figure 4A).

When CXCL16 was induced in GECs by treating them with IL-1 β , peritoneal macrophages adhered, and anti-CXCL16 RS attenuated but did not completely block it (Figure 4B). Taken together, our findings indicate that anchored CXCL16 functions as a cell adhesion molecule for cells that express CXCR6. The finding that the cell adhesion of GECs expressing CXCL16 was not completely blocked with anti-CXCL16 RS indicates that other adhesion molecules are induced in GECs by IL-1 β .

In Vitro Chemotactic Response of Glomerular Inflammatory Leukocytes to CXCL16

To investigate the role of CXCL16 in leukocyte recruitment during anti-GBM GN, inflammatory cells were isolated from nephritic glomeruli on day 7 and 15. The influence of CXCL16 on chemotaxis was examined by transfilter migration assays. Glomerular infiltrates from day 7 or 15 displayed a robust chemotactic response to CXCL16. The maximum migration was in response to 10^{-7} M with less migration in response to 10^{-8} M at both days 7 and 15 (Figure 5A).

Expression of CXCL16/CXCR6 in Nephritic Glomeruli

In vivo expression of CXCL16 in glomeruli was present throughout the development of anti-GBM GN (day 3 to day 60). Normal glomeruli constitutively expressed CXCL16 mRNA, but from day 3 after injection of anti-GBM

Figure 7. *In situ* hybridization of CXCL16 mRNA in the kidney. **A:** Anti-sense CXCL16 was found (brown granules) in glomeruli including crescents and to a lesser degree in tubules. **C:** No signal was found with the sense CXCL16 probe. In **B** and **D**, a black filter was used with sections shown in **A** and **C**, respectively, to demonstrate the pattern of the specific hybridization signal and the background. In **E** and **F**, a lower magnification of two fields is shown to demonstrate CXCL16 expression in glomeruli. The glomerulus with higher hypercellularity (**E**, arrow) is strongly positive.

Ab there was a substantial increase in the CXCL16 mRNA (Figure 5B). Western blot analysis of rat glomeruli revealed CXCL16 protein on day 3 after anti-GBM Ab injection, and expression decreased by day 21 (Figure 5C). Soluble CXCL16 (~35 kD) was the major form, and it correlated with the induction of ADAM10 expression, a disintegrin-like metalloproteinase that cleaves the transmembrane protein to form soluble CXCL16 (Figure 5D).

Concordant with the increased expression of CXCL16, there was an increase in expression of its receptor in nephritic glomeruli (Figure 5B). We next investigated which cells express the chemokine. Macrophages from nephritic glomeruli expressed both CXCL16 (Figure 6A) and CXCR6 (Figure 6B); bone marrow-derived macrophages were also shown to express CXCL16 and CXCR6 (Figure 6, A and B).

Figure 8. Photomicrographs (original magnification, $\times 100$ with a $\times 400$ magnification **inset**) of periodic acid-Schiff-stained glomeruli from anti-GBM GN rats treated with NRS or with anti-CXCL16 RS.

In situ hybridization demonstrated that CXCL16 mRNA was mainly expressed in the glomeruli, including in the crescents. Some segments of the renal tubules had scattered grains over the kidney tubular epithelial cells, suggesting that it also might be present in these cells (Figure 7).

Functional Role of CXCL16 during Anti-GBM GN

To define the functional role of CXCL16 in the development of anti-GBM GN, we induced GN in WKY rats and gave them daily i.p. injections of anti-CXCL16 RS or NRS as a control. On day 8 in the untreated rats, anti-GBM GN led to an infiltration of CD8⁺ T cells and ED1⁺ monocytes/

macrophages; there also was severe endocapillary cell proliferation and glomerular fibrinoid deposition. In rats treated with anti-CXCL16 RS, monocyte/macrophage infiltration was significantly attenuated, and glomerular hypercellularity and glomerular fibrinoid deposition were reduced (Figures 8 to 10). In contrast, CD8⁺ T cell infiltration was unaffected (data not shown). On day 16, antiserum treatment significantly reduced monocyte/macrophage infiltration, glomerular hypercellularity, crescentic formation, and glomerulosclerosis (Figures 8 to 10). As expected, CD8⁺ cells were not observed in either the control- (NRS) or anti-CXCL16 Ab-treated rats because this model of GN is characterized by an early infiltration of CD8⁺ cells (maximum increase on day 3). We also delayed treatment with anti-CXCL16 RS until day

Figure 9. Photomicrographs of glomeruli from anti-GBM GN rats treated with NRS or with anti-CXCL16 RS. ED1⁺ monocytes/macrophages were reduced by treatment with anti-CXCL16 RS.

7 or day 11 after the injection of the anti-GBM Ab and treated rats from day 7 to day 11 or from day 11 to day 15 with anti-CXCL16 RS. Rats in these studies were paired according to the degree of proteinuria so that we could examine the influence of blocking CXCL16 at a similar stage of kidney damage. Rats were euthanized on day 12 or day 16. In rats treated with anti-CXCL16 RS from day 7 to day 11, glomerular hypercellularity and necrotizing damage were reduced 55.3 and 42.7%, respectively (Figures 8 and 10). Chronic tubulointerstitial injury was also significantly reduced (55.6%) (data not shown). The anti-CXCL16 RS also reduced macrophage infiltration to 55.4% of the values found in untreated rats (Figures 9 and 10). Even when the treatment was delayed until day 11 (day 11 to day 15), when the second peak of macrophage infiltration occurs, anti-CXCL16 RS significantly reduced glomerular hypercellularity (49.2%) and necrotizing damage (70.1%) (Figures 8 and 10). The reduction in the severity of the glomerular lesion correlated with a significant decrease in macrophage infiltration (61.6%) (Figures 9 and 10).

Analysis of Renal Function

Anti-CXCL16 treatment also prevented the increase in proteinuria from day 5 onwards in rats treated in the early phase of the disease (Figure 11A). It is important to note that anti-CXCL16 RS treatment also significantly reduced proteinuria in rats treated during the progressive phase of GN, from day 7 to day 11 (Figure 11B) or from day 11 to day 15 (Figure 11C). No difference in the urine volume was found between the groups.

Discussion

Anti-GBM GN in WKY rats is a severe form of crescentic GN characterized by an initial glomerular infiltration of

CD8⁺ T cells and monocytes/macrophages.^{39,53} Our results demonstrated that CXCL16 has an important role in the development of this model of GN. In cultured GECs, we found that CXCL16 is constitutively expressed, but proinflammatory stimuli will induce CXCL16 and enhance the adhesion of macrophages to GECs. These results are consistent with the report of CXCL16-mediated adhesion of cells expressing CXCR6.^{20,54}

When we investigated the role of CXCL16 in recruiting leukocytes during anti-GBM GN, we found robust mRNA expression of CXCL16 and that glomerular infiltrates displayed a robust chemotactic response to CXCL16. Besides the increase in mRNAs, we found the soluble CXCL16 protein (the chemokine) in nephritic glomeruli. Because the disintegrin and metalloproteinase ADAM10 cleaves the transmembrane CXCL16 to form soluble CXCL16, our finding that ADAM10 is induced in glomeruli during anti-GBM GN may explain how the soluble CXCL16 protein increases.^{29,30} Because GECs express CXCL16, our results provide a mechanism by which CXCR6-expressing cells become adherent to the glomerulus and explain how a soluble chemokine is generated, leading to recruitment of macrophages.

Evidence that CXCL16 plays a critical role in GN was uncovered when we blocked CXCL16 with anti-CXCL16 RS during the acute inflammatory phase of the disease (day 1 after the injection of anti-GBM GN, for 7 days). We found decreased glomerular injury and proteinuria along with less infiltration of macrophages. Interestingly, CXCL16 did not prevent accumulation of CD8⁺ cells. Because neutralizing Ab to MIP-1 α can attenuate proteinuria but not the accompanying influx of neutrophils in anti-GBM GN,⁵⁵ expression of redundant chemokines may explain why blocking CXCL16 did not prevent CD8⁺ cell accumulation.^{1,4,56}

Notably, anti-CXCL16 RS administration from day 1 to 7 significantly attenuated macrophage infiltration,

Figure 10. Quantification of ED1⁺ cell infiltration and histological parameters in the glomeruli from anti-GBM GN rats treated with anti-CXCL16 RS or NRS. One hundred glomeruli per section of the kidney of each rat ($n = 8$) was examined; the results are expressed as mean $\pm$ SD. * $P < 0.001$, ** $P < 0.005$, *** $P < 0.05$.

histological damage, and reduced proteinuria measured 16 days after giving the anti-GBM Ab. The conclusion that CXCL16 has a critical role in determining the damage occurring in anti-GBM GN is bolstered by our finding that even delayed treatment with anti-

CXCL16 RS from day 7 to day 11 or from day 11 to day 15 (the first and second peak of macrophage influx, respectively) significantly reduced macrophage infiltration and the severity of the glomerular lesion and improved proteinuria substantially.

Figure 11. Proteinuria in anti-GBM GN rats treated with NRS or anti-CXCL16 RS. **A:** Treatment with anti-CXCL16 RS in the acute phase of anti-GBM GN (treatment started at day 1 of anti-GBM GN for 7 days) decreased proteinuria at each time point. **B:** Treatment with anti-CXCL16 RS in the progressive phase of anti-GBM GN (day 7 to day 11) also decreased proteinuria. **C:** Proteinuria in rats treated with anti-CXCL16 RS in the progressive phase of anti-GBM GN (day 11 to day 15) was also significantly reduced compared with the values of the control group. Results were sampled from eight rats per group ($n = 8$) and expressed as mean $\pm$ SD. * $P < 0.001$, ** $P < 0.007$, *** $P < 0.05$.

These latter results also demonstrated that ongoing expression of CXCL16 is important in the pathogenesis of GN. This conclusion is consistent with the proposal that continued chemokine expression maintains leukocyte influx leading to tubular damage, renal fibrosis, and glomerulosclerosis (progression phase) via release of inflammatory and profibrotic factors.¹⁷

The chemokine MDC/CCL22 is highly expressed throughout anti-GBM GN and attracts macrophages into the glomeruli.¹³ The presence of MDC/CCL22 during the progression of anti-GBM GN could support monocyte/macrophage infiltration and, hence, might explain why neutralization of CXCL16 did not completely block macrophage infiltration into the glomerulus. We recognize that other chemokines (eg, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, and CX₃CL1/fractaline) do promote the inflammatory phase of the disease. Otherwise, our results point to a role of the chemokines MDC/CCL22 and CXCL16 in both early and late phases of this model of glomerulonephritis.

In conclusion, our results suggest that CXCL16/CXCR6 plays a critical role in mediating leukocyte influx during anti-GBM GN in WKY rats. Activation of CXCL16 to promote its expression and its cleavage with release of soluble CXCL16 acts to promote progression damage to glomeruli from early inflammatory as to irreversible kidney damage.

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