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Combinatorial Model of Chemokine Involvement in Glomerular Monocyte Recruitment: Role of CXC Chemokine Receptor 2 in Infiltration During Nephrotic Nephritis

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A sequential model involving chemokines has been proposed for leukocyte extravasation into areas of inflammation; however, site-specific aspects remain to be elucidated. Hence, we studied the role of chemokines produced by mesangial (MC) or glomerular endothelial cells (GEC) and their receptors in glomerular recruitment of monocytes. Stimulation of MC with TNF-α up-regulated mRNA and protein of CC and CXC chemokines but not constitutive expression of the CX3C chemokine fractalkine. While growth-related activity (GRO)-α was immobilized to MC proteoglycans, monocyte chemotactic protein (MCP)-1 was secreted into the soluble phase. Firm adhesion and sequestration of monocytes on activated MC was supported by the GRO-α receptor CXCR2 and to a lesser extent by CX3CR, whereas the MCP-1 receptor CCR2 contributed to their transendothelial chemotaxis toward activated MC. In contrast, fractalkine mRNA and protein was induced by TNF-α and to a lesser extent by CX3CR, whereas the MCP-1 receptor CCR2 contributed to their transendothelial chemotaxis toward activated MC. In conclusion, our results epitomize a combinatorial model in which chemokines play specialized roles in driving glomerular monocyte recruitment and emphasize an important role for CXCR2 in macrophage infiltration during early phases of nephrotic nephritis. The Journal of Immunology, 2001, 166: 5755–5762.

The multistep paradigm of leukocyte trafficking involves sequential and overlapping interactions of signal molecules initiated by selectin-mediated rolling interactions followed by the firm integrin-dependent arrest on Ig ligands (1) and transendothelial migration toward a chemokine gradient (2). This leukocyte cascade can be hierarchically controlled by functionally specialized chemokines, i.e., chemokines can activate integrin adhesiveness to convert rolling into firm arrest or induce directed migration of monocytes (3–7). The functional specificity of chemokines may be associated with differences in their presentation and immobilization to the endothelial surface via heparan-decorated proteoglycans (6–9).

Previous studies have demonstrated the expression of these key molecules in various forms of glomerulonephritis (10). Animal models have shown that the integrin ligands ICAM-1 and VCAM-1 are molecules in various forms of glomerulonephritis (10). Animal models have shown that the integrin ligands ICAM-1 and VCAM-1 are involved in nephrotic nephritis (11, 12). Multiple CC and CXC chemokines are expressed in distinct glomerulopathies, and the CC chemokines monocyte chemoattractant protein-1 (MCP-1; CCL2 according to the new chemokine classification (13) recently proposed) or RANTES (CCL5), as well as the CX3C chemokine fractalkine (CX3CL1), have been implicated in mononuclear cell recruitment during NTN, crescentic glomerulonephritis, or anti-Thy-1 nephritis (14–20). In addition, it has been inferred that the MCP-1 receptor CCR2 participates in early phases of NTN, while the RANTES receptor CCR1, as well as CCR2, appear to increase severity at later stages, suggesting potential anti-inflammatory activities (17, 18). In contrast, blocking CXC chemokines, such as macrophage inflammatory protein (MIP)-2 or growth-related activity (GRO) homologue cytokine-induced neutrophil chemoattractant (CINC), has been revealed to reduce initial neutrophil influx in NTN (19); however, their effect on mononuclear infiltration is not known.

Mononuclear cell infiltration has been identified as an early marker in the pathogenesis of glomerulonephritis (21, 22), and both mesangial cells (MC) and glomerular endothelial cells (GEC) have been involved in their recruitment (10). MC express the CC chemokines MCP-1 and RANTES (23, 24) and the CXC chemokines IL-8 (CXCL8) and GRO (CXCL1) (25–27). In contrast, GEC, which line the capillaries in the glomerulum, exhibit inducible expression of the chemokines RANTES and MCP-1 (28, 29). Moreover, immunofluorescence staining in nephritic but not normal kidneys has demonstrated that fractalkine is expressed in a nonlinear pattern typical of glomerular endothelium (15).

Although leukocyte adhesion and trafficking in the vascular system has been widely studied in vitro and in vivo, less is known about site-specific molecular mechanisms involved in glomerular recruitment. Here we have established a combinatorial model of glomerular monocyte infiltration by investigating the functional specialization of chemokines produced by MC and GEC and the
involvement of their chemokine receptors in different steps of leukocyte recruitment. We found that both fractalkine and GRO-\(\alpha\) via their receptors CX3CR and CXCR2 mediate monocyte arrest on activated GEC, while MCP-1 and CCR2 induce diapedesis across EC, and CXCR2 supports adhesion to residential MC. The relevance of these mechanisms during glomerular infiltration in vivo was confirmed in a telescoped model of NTN where glomerular recruitment and localization of macrophages was almost completely blocked with a CXCR2 antagonist and substantially inhibited with a CCR2 antagonist.

Materials and Methods

**Cell culture and reagents**

Primary human mesangial cells (Clonetics, San Diego, CA) were used at passages 3–9 and cultured in basal MC medium (Clonetics) supplemented with 5% FCS. HUVEC were used at passages 2–5 and grown in low-serum Promocell medium (30). Rat GEC (from H. Holthöfer, Haartmann Institute, University of Helsinki, Helsinki, Finland) were used at passages 7–9 and maintained in RPMI 1640 medium supplemented with 10% FCS. This cell line is transformed by an oncogenic adenovirus, exhibits a highly proliferative phenotype, and consistently expresses endothelial cell markers (31). Tumor necrosis factor-\(\alpha\) stimulated cell types was conducted at 100 U/ml for 4 h. Human blood monocytes were isolated from healthy donors by NycoPrep density gradient centrifugation (Nycomed, Oslo, Norway) and separated from platelets by multiple low-gravity washes, as described previously (3). This protocol resulted in a purity of >85% monocytes and a minimal contamination with platelets (<5%), as assessed by expression of CD14 and P-selectin. The rat macrophage cell line NR8383 was cultured in complete Ham’s F-12 medium, supplemented with 0.5% FCS and penicillin-streptomycin (32).

**RT-PCR**

Total RNA was isolated by phenol/chloroform/isooamylalcohol extraction and cDNA was reverse transcribed from 2 \(\mu\)g RNA. PCR products were analyzed by agarose gel electrophoresis and quantitated by HPLC analysis as described (3, 35–37). Primer sequences were as published for \(\beta\)-actin (33, 34) were provided by Dr. I. Clark-Lewis (University of Massachusetts, Boston, MA), and Met-RANTES was provided by Dr. A. Proudfoot (Seron Pharma Research, Cambridge, MA). Monoclonal Abs to human MCP-1, GRO-\(\alpha\), IL-8, or fractalkine and isotype controls were purchased from Serotec (Oxford, U.K.), BioSource (Camarillo, CA), or R&D Systems (Minneapolis, MN). Chemokines were purchased from PeproTech (Rocky Hill, NJ). Reagents were obtained from Sigma (Deisenhofen, Germany), unless otherwise stated.

**Flow cytometry and calcium mobilization**

Confluent MC were trypsinized, washed, and reacted with saturating mAb concentrations for 30 min on ice, stained with FITC-conjugated IgG (Boehringer Mannheim, Mannheim, Germany), and analyzed in a FACS-cant (Becton Dickinson, Mountain View, CA) (30, 41). Surface expression of CD11b (41, 42) and intracellular calcium mobilization (41) in response to chemokines was studied as previously described, using flow cytometry and fura-2 spectrofluorometry, respectively. To test the cross-species reactivity of chemokines and their antagonists, calcium influx and Mac-1 surface expression was measured in rat macrophages and isolated human blood monocytes stimulated by GRO-\(\alpha\) and MCP-1 derived from the other species and inhibited by GRO-\(\alpha\)-\(\beta\)-antagonist and MCP-1\(\alpha\)-\(\beta\)-antagonist (data not show). This suggested a sufficiently high cross-species reactivity, allowing the use of these peptides in our models.

**Immunofluorescence**

MC were grown to confluence on coverslips. MC were fixed in 2% paraformaldehyde and incubated for 2 h at room temperature (RT) with 10\% heat-inactivated human serum in PBS to block nonspecific binding. To differentiate surface and cytoplasmic staining for MCP-1, some cells were permeabilized in 0.1% saponin for 2 min at RT. Cells were incubated with the primary Ab for 30 min at RT, washed, and then incubated with a FITC-conjugated IgG for 30 min at RT. Some cells were stained with heparitinase (0.5 \(\mu\)M for 1 h at 37°C in HBSS supplemented with 10 mM HEPES, 0.5% human serum albumin, and 2 mM Ca\(^{2+}\)). Coverslips were allowed to air dry, mounted with Mowiol (Calbiochem, Baol Soden, Germany), and analyzed using a Leica (Deerfield, IL) DMRBFE fluorescence microscope with a 100\% oil immersion objective.

**Quantification of MCP-1, GRO-\(\alpha\), IL-8, and fractalkine protein**

MC supernatants were collected and sterile filtered. The concentration of MCP-1 and GRO-\(\alpha\) protein present in the supernatants was determined using a sandwich ELISA (R&D Systems) performed according to the manufacturer’s protocols. IL-8 and fractalkine concentrations were measured, following R&D Systems protocols for a double ligand ELISA.

**Western blotting**

TNF-\(\alpha\) (200 \(\mu\)M, 12 h)-stimulated HUVEC were prepared as reported (43). MC or GEC were treated with TNF-\(\alpha\) (100 \(\mu\)M) for 4 h, supernatants were collected and sterile filtered, and then MC were lysed in sample buffer containing protease inhibitors. Recombinant proteins were diluted in sample buffer. Proteins were separated by 10% SDS-PAGE and transferred to Immobilon membranes (Millipore, Eschborn, Germany) using an electroblotting system. Membranes were blocked for 2 h at RT with 5\% dry milk and 0.05\% Tween 20 (Fikfa, Buchs, Switzerland) in TBS and incubated with anti-fractalkine Ab (0.1 \(\mu\)g/ml). Visualization of bound Abs was conducted with the ECL system (Amershams, Little Chaflont, U.K.).

**Monocyte adhesion in shear flow**

Laminar flow assays were performed as previously described (3, 44). MC, GEC, or HUVEC were grown to confluence in 35-mm petri dishes that were assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an Olympus (New Hyde Park, NY) IMT-2 microscope. Monocytes (0.5 \(\times\) 10\(^6\)/ml) were stimulated or left untreated. Transwells were inserted, and monocytes were added to the top chamber. A dilution of cells served as a measure of input. Cells were allowed to transmigrate for 2 h. Input and migrated cells were counted by flow cytometry with monocyte light scatter gates. Flow cytometry following staining for the monocyte marker CD14 revealed that >90% of cells in the gated cell population were CD14-positive monocytes, thus confirming appropriate light scatter gates for monocytes (data not shown).

**Chemotaxis assays**

Transendothelial monocyte chemotaxis assays were performed as described (3, 42, 45–47). In brief, HUVEC or GEC were grown on 6.5-mm transwell inserts (8 \(\mu\)M pore size; Costar, Cambridge, MA). MC were grown to confluence in the bottom chamber of the 24-well plate and stimulated or left untreated. Transwells were inserted, and monocytes were added to the top chamber. A dilution of cells served as a measure of input. Cells were allowed to transmigrate for 2 h. Input and migrated cells were counted by flow cytometry with monocyte light scatter gates. Flow cytometry following staining for the monocyte marker CD14 revealed that >90% of cells in the gated cell population were CD14-positive monocytes, thus confirming appropriate light scatter gates for monocytes (data not shown).

**Nephrotoxic nephritis model and in vivo transfer of macrophages**

The telescoped model of NTN was induced, and in vivo transfer of fluorescently labeled macrophages was performed essentially as described (32, 48). Male Sprague Dawley rats (purchased from Harlan, Biecker, U.K.), weight 190–250 g, were preimmunized with 1 mg rabbit IgG s.c. followed 1 wk later by injection of 5 ml/kg of rabbit serum containing high titers of anti-rat glomerular basement membrane Abs. NR8383 macrophages were harvested, and cell membranes were fluorescently labeled with 2 \(\mu\)M PKH-26GL, washed in Ham’s F-12, and rested in complete Ham’s F-12 for 24 h before injection. For delivery of macrophages to the left kidney, rats were anesthetized using hypnorm (Jannsen, High Wycombe, U.K.) i.m. (0.3 ml/
kg) and diazepam (Phoenix Pharmaceutics, Gloucester, U.K.) i.p. (2.5 mg/kg) 24 h after induction of NTN. The left renal artery was exposed using a standard anterior approach and directly cannulated using a 27-gauge needle. Fluorescently labeled macrophages (5 × 10^6) preincubated with or without CX,CR mAb (Ref. 20, kindly provided by L. Feng), GRO-α,β,δ or MCP-1p,α,β (3 μg each) in a volume of 0.4 ml for 30 min on ice, were injected over 1 min. Bleeding was stopped by compression of the renal artery, and adequate perfusion of the left kidney was seen before closure of the abdominal wall. Animals were killed 1 day after operation.

Pathology
Glomeruli were isolated from fresh kidney tissue by sieving through 250-μm and 150-μm diameter sieves with collection on a 65-μm sieve (32) or kidney tissue was snap-frozen in isopentane embedded in OCT. Isolated glomeruli were incubated with anti-rabbit IgG-FITC to outline the glomerular basement membrane. Localization of macrophages to inflamed glomeruli was assessed by counting the number of fluorescently labeled macrophages in 150–200 randomly selected glomeruli.

Statistics
Statistical significance was determined by ANOVA, and differences with p < 0.05 were considered to be significant.

Results
TNF-α up-regulates mRNA expression of chemokines in MC
To investigate whether TNF-α stimulation of MC induces mRNA transcriptional activity for chemokines, RT-PCR was performed. Analysis of PCR products showed that expression of mRNA of the chemokines GRO-α, IL-8, and MCP-1 was markedly up-regulated by TNF-α (Fig. 1A). In contrast, mRNA levels of the CX,C chemokine fractalkine were not clearly increased by stimulation of MC with TNF-α (Fig. 1A). These results were confirmed by quantification of the PCR products relative to β-actin using HPLC analysis (Fig. 1B).

TNF-α stimulation induces surface expression of chemokines GRO-α and IL-8 but not fractalkine and secretion of MCP-1 and IL-8
We studied whether up-regulation of chemokine transcripts was associated with an increase in surface protein expression. In accordance with mRNA expression, Western blot and flow cytometric analysis revealed that TNF-α did not affect total and surface protein expression of fractalkine in MC but strongly induced fractalkine expression on HUVEC (Fig. 1, C and D). The anti-fractalkine Ab seemed to be specific as it bound to recombinant chemokine domain of fractalkine but not MCP-1 in Western blots (Fig. 1C). Endothelial activation with TNF-α has been shown to result in surface immobilization of GRO-α, whereas MCP-1 is secreted as a soluble protein (7). In activated MC, IL-8 has been described to associate with the surface but is also released into the soluble phase (25, 26). To detect whether the chemokines GRO-α, IL-8, and MCP-1 are immobilized to the MC surface following their induction, flow cytometric analysis and immunofluorescence were performed. Resting MC showed marginal expression of surface-associated GRO-α, which was markedly increased by TNF-α (Fig. 1, E and F). Treatment with heparitinase abolished staining for GRO-α (Fig. 1F), revealing that binding to heparan proteoglycans is involved in the surface immobilization of GRO-α on MC. Immunofluorescence staining of MC with mAbs to IL-8 and

FIGURE 1. TNF-α up-regulates CC and CXC chemokines but not the CX,C chemokine fractalkine in MC. A. MC were treated with or without TNF-α (100 U/ml) for 4 h, and mRNA expression was analyzed by RT-PCR in comparison to β-actin. Shown are a representative agarose gel (A) and HPLC quantification standardized to β-actin of at least three independent experiments (B). C. HUVEC or MC treated with or without TNF-α for 12 h and 4 h, respectively. Cell lysates were analyzed by Western blot using a polyclonal Ab to fractalkine, and mAb specificity was confirmed by comparison with recombinant fractalkine and MCP-1. Shown is a representative experiment from three independent experiments. D and E. MC were treated with TNF-α for 12 h, and surface protein expression of fractalkine (D) and GRO-α (E) (solid line) in comparison to isotype control (dotted line) was analyzed by flow cytometry and the specific mean fluorescence intensity is given as inserts. F. Immunofluorescence staining of resting or TNF-α-activated MC was performed with mAb to GRO-α or MCP-1. Some MC were treated with heparitinase (0.5 U/ml) or permeabilized. Shown are representative images from three independent experiments. G. MCP-1, GRO-α, and IL-8 were quantitated by ELISA in supernatants of untreated or TNF-α-stimulated MC. Data represent mean ± SD from three experiments performed in duplicate. *, p < 0.05 vs control.
MCP-1 detected a similar pattern for IL-8 (data not shown), but in contrast MCP-1 was not detected on the surface of resting or TNF-α-treated nonpermeabilized MC but in the cytoplasm of stimulated MC following permeabilization (Fig. 1F). Thus, MC indeed produce MCP-1 protein that is not immobilized on the cell surface. To assess whether these chemokines were also released into the soluble phase, MCP-1, GRO-α, and IL-8 in MC supernatants were measured by ELISA. Supernatants of resting MC revealed basal levels of MCP-1, GRO-α, and IL-8. After TNF-α stimulation, the amounts of soluble MCP-1 and IL-8 were markedly increased, while GRO-α was only slightly up-regulated (Fig. 1G). In contrast, fractalkine was not detected in MC supernatants by ELISA or Western blot (data not shown). Thus, activated MC secrete MCP-1 and IL-8 as soluble proteins, immobilize IL-8 and foremost GRO-α on the surface, while fractalkine is constitutively expressed in its membrane form and not inducible.

Increased monocyte adhesion to TNF-α-stimulated MC: involvement of CXCR2

Firm monocyte adhesion on activated endothelium under shear flow involves α4 and β2 integrins interacting with Ig ligands (5, 44, 49). Moreover, surface-bound GRO-α can support CXCR2-dependent monocyte arrest on activated endothelium in shear flow (7). Fractalkine has also been shown to mediate integrin- and PTX-independent firm adhesion of leukocytes to endothelial cells (50). Hence, we tested the role of integrins and chemokines in shear-resistant monocyte arrest to activated MC under flow conditions as a sensitive measure of adhesive strength. TNF-α-activated MC supported spontaneous monocyte arrest (22.8 ± 1.7 cells/mm²) (Fig. 2A), albeit less efficiently than activated endothelium (7). Pretreatment with a combination of α4 and β2 integrin mAbs or with PTX reduced firm arrest of monocytes to background levels supported by resting MC (Fig. 2A), confirming that induced arrest was dependent on integrins activated by signaling via Gαi protein-coupled receptors. Preincubation with the GRO-α8–73 peptide analog, which competes with the binding of GRO-α and IL-8 to their receptor CXCR2, but not the CCR2 receptor antagonist MCP-19–76 (1 μg/ml), or soluble fractalkine (100 nM) for 30 min. The firm arrest of monocytes to MC was determined by counting the number of cells attached in multiple fields after a 4-min period and expressed as cells/mm². Data represent the mean ± SD of at least three independent experiments. B, PBMC treated with or without MCP-19–76 or GRO-α8–73 (1 μg/ml) and allowed to transmigrate across transwell filters seeded with confluent HUVEC toward MC were left untreated or stimulated with TNF-α (100 U/ml) for 4 h in the bottom chamber for 2 h. Migrated monocytes were counted by flow cytometry with appropriate light scatter gates. Data are expressed as mean ± SD of at least three independent experiments. * p < 0.05 vs control.

Transendothelial migration of monocytes toward MC depends on CCR2

Monocyte transmigration following arrest appears to require a soluble chemokine gradient, e.g., MCP-1 (7). To study transendothelial migration of monocytes toward activated MC secreting chemokines, we performed a two-chamber chemotactic assay to mimic the structural configuration of the glomerulus. Monocytes introduced to the top chamber were allowed to transmigrate across HUVEC (Fig. 2B) or GEC (not shown) monolayers grown on filter inserts toward MC in the lower chamber. Transmigration of monocytes was increased ~2-fold by TNF-α stimulation of MC as compared with transmigration elicited by resting MC. This response was significantly inhibited in the presence of MCP-19–76, but only slightly reduced in the presence of GRO-α8–73 (Fig. 2B). Notably, background migration toward resting MC was unaffected by either peptide analog, suggesting that random migration may prevail under these conditions. Hence, transendothelial chemotaxis toward MC is preferentially mediated by CCR2.

Chemokine expression in GEC

The CX2C chemokine fractalkine, which is induced by cytokines in endothelial cells (38), has been involved in leukocyte recruitment during crescentic NTN, where it can be detected in the glomerular endothelium (15). To assess transcriptional regulation of GRO-α, MCP-1, and fractalkine in GEC, cells were stimulated with TNF-α or IL-1β, and RT-PCR with specific primers was performed. Resting GEC expressed fractalkine, GRO-α, and MCP-1 mRNA, which was up-regulated after TNF-α or IL-1β stimulation (Fig. 3A and data not shown). This was confirmed by quantification of PCR products using HPLC analysis (Fig. 3B). Flow cytometric analysis revealed that the transcriptional up-regulation was accompanied by a strong induction of fractalkine surface protein expression as compared with unstimulated GEC (Fig. 3C). In accordance with observations in HUVEC (38), these data confirm that fractalkine expression can be substantially induced by cytokines, such as TNF-α, in GEC. This contrasts our findings in MC,
We then studied monocyte arrest to activated GEC in shear flow. Treatment of GEC with TNF-α markedly increased firm monocyte arrest (20.8 ± 2.0 cells/mm²), which was profoundly diminished by treatment of monocytes with the CXCR2 antagonist GRO-α8–73 but not MCP-1α9–76 (Fig. 3D and data not shown). Moreover, firm arrest was also markedly reduced by preincipulation of monocytes with soluble fractalkine, and a combination of PTX and soluble fractalkine had stronger effects than either substance alone (Fig. 3D). These data suggest that firm monocyte arrest to activated GEC is predominantly mediated by CXCR2 and fractalkine. In contrast, firm monocyte arrest on TNF-α-stimulated HUVEC (92.8 ± 10.8 cell/mm²) was more markedly decreased by pretreatment with PTX or GRO-α8–73 than by soluble fractalkine and was more markedly inhibited by cotreatment with soluble fractalkine and PTX than with either substance alone (Fig. 3E). These data reveal a remarkable difference in the involvement of chemokines in monocyte arrest to endothelial cells from different vascular beds, although it cannot be excluded that this may also reflect species differences.

**CXCR2 and CCR2 antagonists inhibit the localization of macrophages to inflamed glomeruli in vivo**

To study the effects of blocking CXCR2 and CCR2 on glomerular leukocyte recruitment in vivo, macrophage localization was assessed in a rat model of glomerular inflammation caused by experimentally induced NTN. In the control group, the injected macrophages prominently accumulated in inflamed glomeruli of diseased rats as early as 24 h after induction of NTN (Fig. 4, A and B). This infiltration was markedly attenuated when macrophages were pretreated with MCP-1α9–76 and even more substantially inhibited after pretreatment with GRO-α8–73 (Fig. 4, A and B). By contrast, pretreatment of macrophages with a CX3CR mAb (15) was rather ineffective at this early time point, in accordance with glomerular detection of GRO-α and MCP-1 but not endothelial fractalkine, which is expressed and involved at later stages (Ref. 19 and data not shown). Consistent with our findings in vitro, these results show that CCR2 plays an important role in glomerular infiltration. Notably, the more pronounced inhibition of glomerular localization by blocking CXCR2 suggests that the initial arrest triggered by CXCR2 is a crucial prerequisite for subsequent steps of monocyte extravasation, i.e., diapedesis or retention.

**Discussion**

Hypercellularity is an early event in the pathogenesis of glomerular damage, often preceding functional and structural changes (21, 22). Increased adhesion, diapedesis, and accumulation of mononuclear phagocytes is frequently associated with proteinuria and impaired renal function, and the infiltration with immune effector mononuclear cells has been proposed to initiate and maintain renal damage by secretion of inflammatory cytokines, reactive oxygen species, or proteolytic enzymes (51, 52). In vivo studies implicated that TNF-α as a culprit of glomerular dysfunction provides a link between increased glomerular and systemic expression of TNF-α and the perpetuation of glomerular damage (53, 54).
Chemokines and Glomerular Monocyte Recruitment

Chemokines can be immobilized on cell surfaces via heparan sulfate or related glycoproteins or secreted as soluble molecules (7–9). Immunofluorescence staining revealed that upon TNF-α stimulation of MC, GRO-α, and IL-8 but not MCP-1 are presented on the MC surface via binding to heparan proteoglycans, whereas MCP-1 was secreted as a soluble protein. This differential immobilization pattern was associated with a major involvement of the GRO-α receptor CXCR2 in firm monocyte arrest on TNF-α-activated MC, but not the MCP-1 receptor CCR2 and RANTES receptors, as shown by blocking with peptide antagonists. Moreover, blocking IL-8 produced by MC decreased monocyte arrest to activated MC to a lesser extent than blocking CXCR2, suggesting a contribution of both GRO-α and IL-8. Inhibition of CXCR2 also decreased monocyte arrest on activated GEC. Monocytes exhibit higher surface expression of CXCR2 than CXCR1 (56), supporting that CXCR2 which binds both GRO-α and IL-8 is the predominant receptor involved in inducing monocyte arrest. Thus, our data clearly extend the principle that immobilized chemokines, such as GRO-α and IL-8 and their receptor CXCR2, but not soluble chemokines, such as MCP-1, are crucial for mediating firm arrest of monocytes and neutrophils (5, 7).

MCP-1 and its receptor CCR2 may be more important in directing monocyte migration, as observed on activated HUVEC (7). Indeed, CCR2 was predominant in mediating transendothelial chemotaxis toward activated MC, implicating soluble MCP-1 in this process. In contrast, CXCR2 was less important for transmigration in this system, suggesting minor roles for soluble IL-8 and GRO-α. Although chemoattractants can trigger leukocyte adhesiveness both in a soluble or surface-bound form depending on local concentrations achieved (5–7), we have demonstrated that soluble MCP-1 is crucially involved in attraction of monocytes to MC, whereas surface-associated GRO-α and IL-8 mediate subsequent sequestration of monocytes on MC.

The CX3C chemokine fractalkine has recently been detected in nephritic rat glomeruli and has been implicated in leukocyte recruitment in crescentic glomerulonephritis (15). While the soluble chemokine domain of fractalkine is an effective chemotactic agent for leukocytes, fractalkine may also trigger a PTX-insensitive and Ca2⁺⁺- and integrin-independent arrest of cells when immobilized on the cell surface (38, 57–59). We have observed that TNF-α stimulation did not up-regulate expression of fractalkine mRNA and surface-expressed protein in MC. In contrast, activation of GEC led to a marked increase in fractalkine transcription, as has been found with stimulated HUVEC (38). While playing a minor role in monocyte sequestration on MC, fractalkine and CX3CR were involved in shear-resistant monocyte arrest to activated GEC, possibly explaining effects of immunoneutralizing CX3CR in the prevention of crescentic glomerulonephritis (15). Moreover, a combination of PTX and CX3CR desensitization additively inhibited monocyte arrest on GEC, inferring that G protein-independent effects of fractalkine, and G protein-dependent signaling by other chemokines, e.g., GRO-α, both contribute to monocyte arrest on GEC. Together, these data suggest that while fractalkine may act as a supportive, nondiscriminating signal for monocyte adhesion to MC, its interactions with CX3CR may play an integral part in enabling firm monocyte arrest on GEC in shear flow.

However, the site-specific cellular and molecular mechanisms involved in the glomerular recruitment of monocytes in response to TNF-α remain to be elucidated.

Successful leukocyte emigration is mediated by the overlapping actions of multiple signal molecules that are expressed in glomerulopathies and are known to be inducible by TNF-α (10–12, 14–20, 23–29). Here we confirm previous findings that TNF-α markedly up-regulated the expression of the chemokines GRO-α, IL-8, and MCP-1 (10, 23, 25–27). Notably, the up-regulation of these chemokines resulted in an increase in shear-resistant monocyte adhesion to TNF-α-stimulated MC. Monocyte arrest was significantly inhibited by PTX, indicating that integrin activation by signaling via Gα protein-coupled receptors is involved as seen with lymphocyte arrest on high endothelial venules (55).

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MCP-1 and its receptor CCR2 may be more important in directing monocyte migration, as observed on activated HUVEC (7). Indeed, CCR2 was predominant in mediating transendothelial chemotaxis toward activated MC, implicating soluble MCP-1 in this process. In contrast, CXCR2 was less important for transmigration in this system, suggesting minor roles for soluble IL-8 and GRO-α. Although chemoattractants can trigger leukocyte adhesiveness both in a soluble or surface-bound form depending on local concentrations achieved (5–7), we have demonstrated that soluble MCP-1 is crucially involved in attraction of monocytes to MC, whereas surface-associated GRO-α and IL-8 mediate subsequent sequestration of monocytes on MC.

The CX3C chemokine fractalkine has recently been detected in nephritic rat glomeruli and has been implicated in leukocyte recruitment in crescentic glomerulonephritis (15). While the soluble chemokine domain of fractalkine is an effective chemotactic agent for leukocytes, fractalkine may also trigger a PTX-insensitive and Ca2⁺⁺- and integrin-independent arrest of cells when immobilized on the cell surface (38, 57–59). We have observed that TNF-α stimulation did not up-regulate expression of fractalkine mRNA and surface-expressed protein in MC. In contrast, activation of GEC led to a marked increase in fractalkine transcription, as has been found with stimulated HUVEC (38). While playing a minor role in monocyte sequestration on MC, fractalkine and CX3CR were involved in shear-resistant monocyte arrest to activated GEC, possibly explaining effects of immunoneutralizing CX3CR in the prevention of crescentic glomerulonephritis (15). Moreover, a combination of PTX and CX3CR desensitization additively inhibited monocyte arrest on GEC, inferring that G protein-independent effects of fractalkine, and G protein-dependent signaling by other chemokines, e.g., GRO-α, both contribute to monocyte arrest on GEC. Together, these data suggest that while fractalkine may act as a supportive, nondiscriminating signal for monocyte adhesion to MC, its interactions with CX3CR may play an integral part in enabling firm monocyte arrest on GEC in shear flow.
While the expression of MCP-1, MIP-1, CINC, RANTES, or fractalkine is profoundly increased, immunoneutralization of CXC,CR, MIP-1, or CINC have been shown to suppress leukocyte infiltration and proteinuria in early phases of NTN (10, 14, 15, 19, 20). In vivo experiments in a teesumpted rat model of NTN further confirmed a combinatorial involvement of chemokine receptors in glomerular recruitment of monocytes/macrophages. The induction of calcium influx in rat macrophages was stimulated by human GRO-α and was inhibited by GRO-α5–73, suggesting a sufficient homology and reactivity for cross-species studies. This extends previous findings that the human RANTES receptor antagonist Met-RANTES effectively reduces renal transplant rejection in a rat model (60). Glomerular macrophage infiltration in the early phase of NTN was markedly reduced by antagonizing CCR2 and even more drastically inhibited by antagonizing CXCR2, which is expressed by activated macrophages (61). Together with the in vitro data, this implies a sequential action of these chemokine receptors in which CXCR2-triggered arrest to inflamed endothelium is the initially critical and rate-limiting event prerequisite to subsequent migration. Notably, in vivo macrophage localization was considerably more inhibited by GRO-α5–73 than by blocking β2 integrins or their ligands and is consistent with an additive effect of PTX and GRO-α in inhibiting arrest in vitro (D. C. Kluth, A. Zernecke, C. Weber, and A. J. Rees, unpublished data). This indicates that GRO-α may act through PTX-sensitive activation of integrin binding but may also serve as a direct adhesive bond as has been suggested for fractalkine. Our results extend findings in knockout models revealing an important role for CXCR2 in macrophage infiltration of atherosclerotic lesions (62) and demonstrate for the first time the crucial involvement of CXCR2 and its ligands in the inflammatory recruitment during glomerulonephritis.

The inhibition of inflammatory recruitment in our NTN model by the CCR2 antagonist was consistent with previous data showing that a MCP-1 Ab can suppress leukocyte infiltration and ameliorate proteinuria in the early phases of murine NTN (14) and that a CCR2 knockout exerts protective effects on day 1 of an accelerated murine NTN model (18). Notably, increased severity was observed at day 3 both in CCR1 and CCR2 knockout mouse models (17, 18). By contrast, CXCR3 Ab treatment was less effective at day 1 than at later time points in our experiments. This is suggestive of a preferential involvement of varying key chemokines during distinct stages during disease progression, i.e., GRO-α and MCP-1 may be crucial at early stages, while fractalkine may become relevant at later time points. Alternatively, fractalkine may act to attract cells from the less marginal circulation to the proximity of the endothelial surface, where highly inducible and immobilized chemokines acting via CXCR2 provide the ultimate signal for firm arrest. Finally, it may be conceivable that an early engagement of a specific receptor results in a pro- or anti-inflammatory commitment that may only become effective in subsequent disease stages.

Our results indicate that a sequence of combinatorial mechanisms enacted by chemokines and their receptors drive glomerular monocyte infiltration. While fractalkine and GRO-α may synergize to mediate initial firm arrest of monocytes on GEC, soluble gradients of MCP-1 and possibly IL-8 established and maintained by secretion of underlying MC direct transendothelial diapedesis of monocytes toward MC, and presentation of immobilized GRO-α and IL-8 support the localization and sequestration of monocytes on MC in the glomerular compartment. This is the first concept of a site-specific multistep model for the regulation of inflammatory monocyte recruitment by functionally specialized chemokines in renal disease and may help envision more finely tuned applications for chemokine (receptor) antagonists to target specific physiological processes.

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References


