CXCR3 Is Involved in Tubulointerstitial Injury in Human Glomerulonephritis

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Chemokines play pivotal roles in the recruitment of inflammatory cells into the kidney. The chemokine receptors CXCR3 and CCR5 are expressed on activated T lymphocytes, and expression of CXCR3 by mesangial cells has been suggested. Detailed description of CXCR3 expression might form a rational basis for use as a diagnostic marker and for therapeutic CXCR3 targeting in human glomerulonephritis. We studied the expression of CXCR3 in renal biopsies by immunohistochemistry (n = 45), and real-time RT-PCR (n = 78). Biopsies were from patients with IgA nephropathy, lupus nephritis, and membranoproliferative glomerulonephritis. Furthermore, cultured human mesangial cells (HMC) were studied for CXCR3 expression, and for functional responses to the ligands CXCL10/IP-10 and CXCL9/Mig. CXCR3-positive cells were rarely found in glomerular tufts, but formed a major part of the tubulointerstitial infiltrates. Consistently, CXCR3 mRNA expression was too low to be quantified in glomerular compartments, and was not detectable in HMC. The published staining for CXCR3 of mesangial cells could be traced to cross-reactivity of an antibody for CXCR3 with a potentially related chemokine receptor as revealed by FACS analysis. Despite an absence of CXCR3 expression, mesangial cells reacted to CXCR3 ligands by proliferation and migration, which was blocked by pertussis toxin but not by an anti-CXCR3 antibody. These results indicate that HMC do not express the classical CXCR3, but may potentially express a related receptor with shared ligand specificity. By immunohistochemistry the number of CXCR3-positive cells, mainly interstitial T cells, correlated with renal function, proteinuria, and percentage of globally sclerosed glomeruli. A significant morphological and numerical correlation between CD3, CXCR3, and CCR5-positive cells indicated a CXCR3/CCR5 double-positive T cell population. No apparent difference in the CXCR3 expression pattern was found between disease entities. CXCR3 expression was localized to interstitial T cells, and these cells correlated strongly with important prognostic markers. Therefore interstitial CXCR3, as well as CCR5-positive T cells might play an important role during progressive loss of renal function, and are potential therapeutic targets in human glomerular diseases. (Am J Pathol 2004, 164:635–649)

Chemokines are members of a family of chemotactic cytokines. As the first chemoattractants specific for subsets of inflammatory cells, chemokines revolutionized our understanding of mononuclear cell recruitment, inflammatory processes, and microenvironment formation. The importance of chemokines during renal inflammation has been described in various studies that have demonstrated expression of chemokines, infiltration of tissue by chemokine receptor-bearing cells, and the therapeutic impact of chemokine receptor antagonists.

The chemokine receptor CXCR3 signals in response to the chemokines CXCL9/monokine induced by γ-interferon (Mig), CXCL10/γ-interferon-inducible protein-10 (IP-10), and CXCL11/interferon-inducible T cell-α chemotactant (I-TAC), which can be released by renal...

Supported by grants from the Else-Kröner Fresenius Stiftung, Bad Homburg an der Höhe, Germany (to S.S.) and the Deutsche Forschungsgemeinschaft (grant BA 2137/1–1 to B.B. and D.S. and grants GK728/7 and SFB 405/B10 to H.-J.G.) and by grants DHGPO1KW9922/2 and the Else-Kröner Fresenius Stiftung, Bad Homburg an der Höhe, Germany (to M.K.).

The study was performed in the framework of the EU-QCG1–2002–01215 grant Chronic Kidney Disease Consortium.

The members of the European Renal cDNA Bank (ERCB) are: H. Schmid, C. D. Cohen, M. Kretzler, D. Schlöndorff (Munich); F. Delarue, J. D. Sraer (Paris); M. P. Rastaldi, G. D’Amico (Milano); P. Doran, H. R. Brady (Dublin); M. Saleem, Bristol; D. Möns, C. Warner, Würzburg; A. J. Rees (Aberdeen); F. Strutz, G. Müller, Göttingen; H.-J. Groene, M. Zeier, E. Ritz (Heidelberg); P. Mertens, J. Floege, Aschen; N. Braun, T. Risler (Tübingen); L. Gesualdo, F. P. Schena (Rari); J. Gerth, G. Stein, Jena; R. Oberbauer, D. Kerjaschki (Vienna); M. Fischereider, B. Krämer, Regensburg; W. Land (Munich-GH); H. Peters, H. H. Neumayer (Berlin); K. Ivens, B. Grabensee, Düsseldorf; F. Mompaso (Madrid); and M. Tesar (Prague).

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Accepted for publication October 27, 2003.

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cells. For example, CXCL10/IP-10 can be expressed by mesangial cells, endothelial cells, and interstitial cells after stimulation with proinflammatory cytokines (especially γ-interferon) or lipopolysaccharide in vitro. Migration of activated peripheral blood lymphocytes toward supernatants derived from cultured tubular epithelial cells was inhibited to a significant extent through blockade of CXCR3.

Expression of the receptor CXCR3 has been demonstrated on circulating T cells with a strong induction of CXCR3 after T cell activation. The receptor has also been described on B cells and natural killer cells. In vitro studies indicate that CXCR3 is predominantly expressed by T helper cells type 1 (Th1). Several studies support that CXCR3 and its corresponding ligands play a pivotal role during inflammatory diseases and allograft rejection. The diseases include inflammatory bowel disease, inflammatory skin diseases, multiple sclerosis, and periodontal disease. The potential role of CXCR3 in allograft pathology has been demonstrated for liver, heart, and lung allografts, both in animal models as well as in human allografts.

We previously studied the expression of the chemokine receptor CCR5 in human kidney biopsies which is also mainly expressed by T cells. In these studies, the number of CCR5-positive interstitial infiltrating cells increased in patients with impaired renal function. CCR5-positive T cells may play a role in chronic transplant nephropathy as patients deficient in CCR5 have an improved long-term allograft survival.

The available data on the potential role of CXCR3-positive cells in renal diseases are still scarce. CXCR3 expression has previously been studied using the anti-human CXCR3 monoclonal antibody (R&D Systems, Minneapolis, MN) on cryostat sections of renal biopsies by real time RT-PCR. Finally, we evaluated the expression of CXCR3 and functional responses to the ligands by mesangial cells in culture. We report that expression of CXCR3 mRNA and its ligands was studied in microdissected tubulointerstitial areas from renal biopsies. To further define the role of CXCR3-positive cells in human glomerulonephritis, we tested two monoclonal antibodies on formalin-fixed, paraffin-embedded tissues (49801.111, R&D Systems, Minneapolis, MN, and 1C6, BD Biosciences Pharmingen, Heidelberg, Germany). Only one turned out to be suitable (1C6, BD Biosciences Pharmingen), on formalin-fixed, paraffin-embedded renal biopsies. The number of CXCR3-positive cells was correlated with histological and clinical data, as well as with the number of CCR5-positive cells. Furthermore, expression of CXCR3 mRNA and its ligands was studied in microdissected tubulointerstitial areas from renal biopsies by real time RT-PCR. Finally, we evaluated the expression of CXCR3 and functional responses to the ligands by mesangial cells in culture. We report that the main CXCR3 expression in human glomerulonephritis is on interstitial infiltrating T cells, the number of which correlates inversely with renal function and histopathology, indicating that CXCR3-positive T cells seem to play an important role in the progression of glomerular diseases.

Materials and Methods

Renal Biopsies

A total of 45 human renal biopsies were included in the study. The study population consisted of biopsies from patients with IgA nephropathy (n = 26), lupus nephritis (n = 12), and membranoproliferative glomerulonephritis (n = 7, Table 1). Laboratory data (serum creatinine, blood urea nitrogen (BUN), and proteinuria) were provided through the clinical data sheet attached to renal biopsy sample. Human biopsies were used following the guidelines of the Ethics Committee of the Medical Faculty of the University of Heidelberg, Germany.

Renal biopsies were fixed in phosphate-buffered 4% formalin, and embedded in paraffin following protocols for routine procedures. In every case the diagnosis was based on light microscopy, immunohistochemistry, and electron microscopy.

Table 1. Clinical Data of the Study Population

<table>
<thead>
<tr>
<th>n</th>
<th>Gender</th>
<th>Age years (range)</th>
<th>Creatinine mg/dl</th>
<th>BUN mg/dl</th>
<th>Proteinuria g/24 hours</th>
<th>Hematuria &gt;10 erythrocytes/µl</th>
<th>Leukozyturiya &gt;10µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>26</td>
<td>9/17</td>
<td>45 (15–76)</td>
<td>2.3 (±0.4)</td>
<td>22 (±8.4)</td>
<td>11/16</td>
<td>2/16</td>
</tr>
<tr>
<td>SLE</td>
<td>12</td>
<td>10/2</td>
<td>30 (22–46)</td>
<td>2.5 (±1.0)</td>
<td>11 (±6.6)</td>
<td>2.8</td>
<td>6/9</td>
</tr>
<tr>
<td>MPGN</td>
<td>7</td>
<td>3/4</td>
<td>48 (16–67)</td>
<td>1.5 (±0.2)</td>
<td>5 (±8.6)</td>
<td>5/0</td>
<td>2/6</td>
</tr>
</tbody>
</table>

IgA, IgA nephropathy; SLE, lupus nephritis; MPGN, membranoproliferative glomerulonephritis; age, years, range; hematuria and leukozyturiya, cases with >10 cells/µl/cases with available data.
Study Population

Renal biopsies from patients with IgA nephropathy, lupus nephritis, and membranoproliferative glomerulonephritis were included in this study (Table 1). The group with IgA nephropathy included 26 renal biopsies and represented the spectrum of the disease. The serum creatinine ranged from normal values (0.7 mg/dl) to 6.5 mg/dl. The percentage of the interstitial area involved in chronic interstitial injury ranged accordingly from 0 to 62% and the percentage of globally sclerosed glomeruli from 0 to 63%. Two biopsies revealed glomerular extracapillary proliferations.

Renal biopsies from patients with lupus nephritis were graded as class III and class IV (six cases each). The glomerular activity scores ranged from 6 to 24 (maximum value 24), and the tubulointerstitial chronicity scores from 0 to 4 (maximal score of 6 corresponding to 100% chronic tubulointerstitial damage). The percentage of the interstitial area involved in chronic interstitial injury ranged from 1 to 72%. Globally sclerosed glomeruli were present in three cases (38% to 55%). Crescents were detected in eight biopsies.

Seven renal biopsies were from patients with membranoproliferative glomerulonephritis. The available serum creatinine ranged between 0.7 and 3.1 mg/dl, and the percentage of globally sclerosed glomeruli between 0 to 67%. Involvement of the interstitium by chronic lesions ranged from 3% to 51% of the biopsy.

Immunohistochemistry

Serial 4-μm sections of renal biopsies were used in the study. Immunohistochemistry was performed as previously described. Tissues were dewaxed in xylene, and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Antigen retrieval was performed in Antigen Retrieval Solution (Vector, Burlingame, CA) in an autoclave oven. Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector). Incubation of the primary antibodies for 1 hour was followed by incubation with biotinylated secondary antibodies (anti-mouse IgG, anti- rat IgG, Vector), and the ABC reagent (Vector). Between incubation steps the slides were washed in phosphate-buffered saline (PBS). 3’3’-diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) was used as detection system. Slides were counter-stained with methyl green, dehydrated, and mounted in Histomount (Zymed Laboratories, San Francisco, CA).

For the detection of CXCR3, two monoclonal antibodies were tested extensively on formalin-fixed, paraffin-embedded tissues (eg, human tonsils). The monoclonal anti-human CXCR3 antibody (clone: 1C6, BD Biosciences Pharmingen), resulted in a reliable staining pattern. This antibody was used at 10 μg/ml in 10% non-fat dry milk. Additionally, the slides were treated for 10 minutes with blocking solution (ID Labs Biotechnology, Ontario, Canada) before the primary antibody was applied.
study for gene expression analysis in renal biopsies (European Renal cDNA Consortium (ERCB)). Informed consent was received according to the guidelines of the respective local ethical committees of the participating centers.

Patients were stratified according to their histological diagnosis by a reference pathologist. Microdissected tubulointerstitial compartments from 78 patients with IgA nephropathy ($n = 52$), lupus nephritis (class II, $n = 3$; class III, $n = 6$; and class IV, $n = 10$), membranoproliferative glomerulonephritis ($n = 7$), and six controls (pre-transplant biopsies) were analyzed. Selected glomerular compartments were analyzed in biopsies from controls, IgA nephropathy, and lupus nephritis ($n = 10$).

The protocols for microdissection and total RNA isolation have previously been described in detail. All primers and probes were obtained from Applied Biosystems. Commercially available pre-developed TaqMan assay reagents (PDARs) were used for the internal standards human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18 S ribosomal RNA (18S rRNA), as well as for human CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC. For human CXCR3 RNA (18S rRNA), as well as for human CXCL9/Mig, and CXCL10/IP-10 (all from R&D Systems GmbH, Wiesbaden, Germany), Cells were harvested through trypsin treatment and total RNA was prepared as described. For each experiment at least six wells were analyzed per experimental condition and time point.

**Real Time RT-PCR**

Real time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems) as previously described. All primers and probes were obtained from Applied Biosystems. Commercially available pre-developed TaqMan assay reagents (PDARs) were used for the internal standards human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18 S ribosomal RNA (18S rRNA), as well as for human CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC. For human CXCR3 (NM_001504) forward and reverse primers (sense TG-GCCGAGAAGACGG, antisense AGGCCAAGAG-CAGCATC), and the internal probe (AGACGTGCG-CAAGTCGGTCACCTC) were designed using Primer Express 1.5 Software (Applied Biosystems, Foster City, CA).

Quantification of the given templates was performed according to the standard curve method. Serial dilutions of standard cDNA from a human nephrectomy were included in all PCR runs and served as standard curve. This method minimizes the influence of inter-assay and inter-run variability. The primers for GAPDH and CXCL10/IP-10 were cDNA-specific, not amplifying genomic DNA. Contamination of genomic DNA was negligible (below 0.1%), as previously demonstrated for the above RNA preparation on microdissected renal specimen. All measurements were performed in duplicate. Controls consisting of distilled H$_2$O were negative in all runs.

**Cell Culture and Functional In Vitro Assays**

The mesangial cell line (HMC) used in this study has previously been described in detail. Primary human mesangial cells were obtained from Clonetics Corp. (San Diego, CA). The immortalized HMC were grown in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany), which was supplemented with 10% bovine serum. HMC were stimulated with human recombinant tumor necrosis factor-$\alpha$ (TNF-$\alpha$, 20 ng/ml), human recombinant interleukin-1$\beta$ (IL-1$\beta$, 2 ng/ml), human recombinant interferon-$\gamma$ (IFN-$\gamma$, 10 ng/ml), either alone or in combination, as well as by basic fibroblast growth factor (bFGF, 5 ng/ml), interleukin-2 (IL-2, 0.25 ng/ml), CXCL9/Mig, and CXCL10/IP-10 (all from R&D Systems GmbH, Wiesbaden, Germany). Cells were harvested through trypsin treatment and total RNA was prepared as described. For each experiment at least six wells were analyzed per experimental condition and time point.

**Flow Cytometry Analysis (FACS) of CXCR3**

For FACS analysis, HMC were collected after detachment with 10 mmol/L EDTA in PBS (pH 8.0), and stained for CXCR3 using two monoclonal antibodies (clone: 1C6, BD Biosciences Pharmingen; clone: 49H101.111, R&D Systems). The FACSscan flow cytometer (Becton Dickinson, USA) was used as previously described. Isotype immunoglobulins served as negative controls.

**RNase Protection Assay and RT-PCR Analysis**

Protection assays were performed according to the manufacturer’s instructions as previously described. Template sets (hCR6) for human chemokine receptors were obtained from BD Biosciences Pharmingen (San Diego, CA). For studying CXCR3 expression 20 ng total RNA prepared from HMC were used per experimental condition, equal amounts of mRNA served as control to exclude incomplete digestion of the probes. After polyacrylamide gel electrophoresis, protected fragments were analyzed using a Storm 840 PhosphorImaging System (Amersham Biosciences Corp. NJ). The protocols for the semi-quantitative reverse transcriptase (RT)-PCR have been described previously (for real-time RT-PCR see above). Combinations of three sense and five antisense primers from the CXCR3 sequence (GenBank Accession No. NM_001504) were used. The primer sequences were as follows. Sense 1: CCATGG TCCT- TGGTGTA; sense 2: GCCCTCTACAGCTCCTCTT; sense 3: TCACC TGCCCTGGTCTG; antisense 1: TGT- TCAGGTAGCGGTCAAAGC; antisense 2: ACAGCTAG- GTGGAGCAGGAA; antisense 3: CCGAACTTGCACC- TACAA; antisense 4: GTCCAGCAGAGGGCACAG; antisense 5: TGTTGGGAAGTTGTATTGGCA. The new splice
variant CXCR3-B would be detectable with the used primers.\textsuperscript{24}

**Chemotaxis Assay**

HMC were plated onto Transwell filter inserts (8-µm pore size, Costar GmbH, Bodenheim, Germany) for 4 hours. In blocking experiments the cells were incubated in assay medium supplemented with either pertussis toxin 1 µg/ml or blocking anti-CXCR3 antibody 1C6 for the last 1.5 hours (azid-free preparation, No. 557 184, BD Biosciences Pharmingen). Afterwards, the filter inserts were transferred to 24-well tissue culture plates with assay medium consisting of DMEM, 0.1% bovine serum albumin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 10 mmol/L Hepes (Invitrogen Life Technologies) supplemented with or without chemokines. To analyze migration toward the chemokine gradient, cells were incubated for 4 hours at 37°C, with CXCL9/Mig or CXCL10/IP-10. Transmigrated HMCs at the bottom of the filter and/or the bottom of the chamber were collected by trypsinization, and counted using a cell counter as previously described.\textsuperscript{30}

**Results**

**CXCR3-Positive Cells Are Rare in Glomeruli from Biopsies with Glomerulonephritis**

The monoclonal antibody 1C6 resulted in a very reproducible, strong staining pattern on formalin-fixed, paraffin-embedded tissue (Figure 1 B, E, and H) after heat pretreatment, whereas the isotype controls were negative (Figure 1, A, D, and G).

In the studied biopsies the number of CD3-positive T cells, CXCR3-positive cells, and CCR5-positive cells was very low within glomerular tufts (Table 2). There was no significant difference between the mean glomerular cell numbers between the three entities. The distribution pattern of CXCR3-positive cells in glomeruli was not consistent with an expression of CXCR3 by a significant part of the mesangial cells. A slightly higher number of CCR5-positive cells was found in lupus nephritis than in the two other entities.

**Mesangial Cells Do Not Express the Classical CXCR3 in Culture**

Romagnani et al.\textsuperscript{22} previously demonstrated that a monoclonal anti-CXCR3 antibody (49801.111) stains primary human mesangial cells in culture, and stains the mesangial area in proliferative glomerular diseases on frozen sections. Consistently, we previously noted positive staining with this antibody on mesangial areas in developing glomeruli of frozen fetal human tissue.\textsuperscript{23} These results differ with the negative staining of the mesangium in fixed biopsy tissue using the 1C6 antibody for immunohistochemistry. To further evaluate the expression of CXCR3 within glomeruli, we used two approaches, ie, real time RT-PCR on RNA extracted from microdissected glomeruli from renal biopsies, as well as in vitro experiments on HMC.

In a series of biopsies from control and diseased kidneys the glomerular mRNA expression for CXCR3 was too low to allow quantification arguing against glomerular CXCR3 expression \( (n = 10) \). HMC were investigated for CXCR3 mRNA expression by RT-PCR (Figure 2), real time RT-PCR, as well as by RNase protection assay (not shown). With neither of these highly sensitive approaches could we demonstrate CXCR3 mRNA expression in HMC although the appropriate control experiments, with mRNA from peripheral blood mononuclear cells (PBMCs) and genomic DNA prepared from HMC were positive (Figure 2, C and D). Furthermore, primary human mesangial cells used for immortalization did not demonstrate CXCR3 expression by RT-PCR (Figure 2E, lanes 3 and 4) or by FACS analysis using the antibody 1C6 (data not shown).

CXCR3 protein on HMC was investigated by FACS analysis comparing the two monoclonal antibodies (Figure 2, A and B). A positive signal was found only with the clone 49801.111 (R&D Systems), whereas the clone 1C6 (BD Biosciences Pharmingen) revealed no signal, directly illustrating differences in these two antibodies (Figure 2, A and B). The HMC remained negative for CXCR3 using the 1C6 antibody when the cells were incubated with the proinflammatory cytokines tumor necrosis factor \( \alpha \), interleukin-1β, and interferon-γ, alone or in combination (data not shown). Additionally, interleukin-2 and β fibroblast growth factor did not induce CXCR3 expression by HMC as evaluated by RT-PCR (Figure 2E). Cytokine treatment had no effect on the positive FACS signal for CXCR3 obtained with the monoclonal antibody 49801.111 (data not shown).

Mesangial cells in culture have been reported to react with calcium influx and increased proliferation on stimulation with the CXCR3 ligands CXCL10/IP-10 and CXCL9/Mig.\textsuperscript{22} Potential responses to CXCL10/IP-10 and CXCL9/Mig on HMC were evaluated by chemotaxis and proliferation assays (Figure 3). HMC demonstrated increased proliferation in response to both chemokines in a dose-dependent manner (Figure 3, A and B). Transwell migration assays revealed a dose-dependent migration of HMC toward a gradient for CXCL9/Mig, CXCL10/IP-10, but not eotaxin/CCL11 (Figure 3, C and D). Thus HMC respond to CXCR3 ligands in the absence of mRNA for CXCR3. The migration of HMC toward the CXCR3 ligands was significantly reduced by pertussis toxin implying a \( \mathrm{G}_{\alpha} \)-coupled receptor in the signal transduction (Figure 3E). Incubation with the antibody 1C6 had no effect on migration (Figure 3E).

Potentially the effects of the CXCR3 ligands on HMC might be mediated by a variant of CXCR3, or a different chemokine receptor, that cross-reacts with the monoclonal antibody 49801.111, but not with 1C6. It is unlikely that these effects could be mediated by the very recently described CXCR3-B splice variant, as this receptor was not present on human mesangial cells, and has been reported to mediate antiproliferative effects in response to CXCL9/Mig and CXCL10/IP-10. Furthermore, CXCR3-B failed to induce cell migration.\textsuperscript{24} We were un-
Figure 1. Immunohistochemistry of renal biopsies from patients with IgA nephropathy (A–C), lupus nephritis (D–F), and membranoproliferative glomerulonephritis (G–I) using an isotype control IgG (A, D, G), the monoclonal anti-human CXCR3 antibody 1C6 (B, E, H), and the monoclonal anti-human CCR5 antibody MC5 (C, F, I) on consecutive tissue sections (original magnification, \( \times200 \)). Please note the strong staining of interstitial infiltrating cells (eg, in E and F in a similar distribution), but the absence of glomerular staining. Panels G–I illustrate a renal biopsy from a patient with membranoproliferative glomerulonephritis with a well-conserved renal interstitial architecture. Only scattered infiltrating cells in the interstitium (arrow) stain positive for CXCR3 and CCR5. In none of the biopsies was there staining of intrinsic cells in the glomerulus.

Table 2. Mean Cell Number According to Morphological Entities

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th></th>
<th></th>
<th>CXC R3</th>
<th></th>
<th></th>
<th>CCR5</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>per HPF</td>
<td>( n )</td>
<td>per Glom</td>
<td>( n )</td>
<td>per HPF</td>
<td>( n )</td>
<td>per Glom</td>
<td>( n )</td>
</tr>
<tr>
<td>IgA</td>
<td>25</td>
<td>11.6</td>
<td>((\pm2.2))</td>
<td>23</td>
<td>0.31</td>
<td>((\pm0.12))</td>
<td>25</td>
<td>17.3</td>
<td>((\pm3.0))</td>
</tr>
<tr>
<td>SLE</td>
<td>11</td>
<td>14</td>
<td>((\pm4.7))</td>
<td>12</td>
<td>0.31</td>
<td>((\pm0.08))</td>
<td>12</td>
<td>15.0</td>
<td>((\pm4.0))</td>
</tr>
<tr>
<td>MPGN</td>
<td>6</td>
<td>15.1</td>
<td>((\pm5.4))</td>
<td>7</td>
<td>0.28</td>
<td>((\pm0.09))</td>
<td>7</td>
<td>16.5</td>
<td>((\pm4.5))</td>
</tr>
</tbody>
</table>

IgA, IgA nephropathy; SLE, lupus nephritis; MPGN, membranoproliferative glomerulonephritis.
able to detect mRNA expression of CXCR3 splice variants in HMC using various CXCR3 primer pairs (Figure 2, C and D).

The Chemokine Receptors CXCR3 and CCR5 Are Mainly Expressed by T Cells Infiltrating the Tubulointerstitial Compartment

In contrast to the low number of CXCR3-positive cells in glomerular tufts, the tubulointerstitial infiltrates were the main site of CXCR3 and CCR5 expression (Figure 4, B, E, and F). The number of positive cells ranged from a few scattered positive cells (Figure 1, B and C) in cases with well-preserved tubulointerstitium, to a high number of CXCR3-positive cells in cases with severe tubulointerstitial injury (Figure 4, A, B, E, and F). The number of CXCR3-positive cells outnumbered CCR5-positive cells (Figure 1, E and F and Figure 5, B and C). CXCR3 and CCR5-positive cells commonly clustered around Bowman’s capsule (Figure 1, E and F and Figure 5, B and C). This was particularly true for partially or globally sclerosed glomeruli. Areas of larger infiltrates (Figure 5, A to D), at times with a nodular appearance, were almost uniformly positive for CXCR3 and CD3, but contained only a relatively low number of CCR5-positive cells, indicating that these sites differ in their cellular composition to those of the diffuse infiltrates. No expression of CXCR3 and CCR5 was apparent on intrinsic renal structures, ie, tubular or glomerular cells (Figure 1, B, C, and F). There was no obvious difference in the pattern of CXCR3-positive cell infiltrates in the various disease entities. Furthermore, the distribution corresponded largely to that of CD3-positive T cells, which was confirmed by double-immunofluorescence staining of select renal biopsies (Figure 6), as well...
as tonsils and transplant nephrectomies (not shown). Replacing the monoclonal antibodies against CXCR3 and CD3 by isotype-matched IgGs resulted in staining for only the respective antibody, excluding cross-reactivity of secondary reagents, or no staining when both antibodies were replaced (Figure 6, A to H). As illustrated in Figure 6, an almost identical distribution of CXCR3 and CD3-positive T cells was found (arrows in Figure 6, M and N). The technical approaches used (serial sections as well as double-immunofluorescence) cannot exclude a small population of other cells (eg, monocytes/macrophages) expressing CXCR3 or CCR5, but the main population are interstitial CD3-positive T cells.

To further support the immunohistological data a total of 84 renal biopsies were dissected into glomerular and tubulointerstitial compartments for real time RT-PCR analysis. As noted above the glomerular expression from selected control and nephritis biopsies was too low to allow reliable detection. CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC, and CXCR3 mRNA expression was studied in tubulointerstitial compartments of renal biopsies from IgA nephropathy (n = 52), lupus nephritis (n = 19), membranoproliferative glomerulonephritis (n = 7), and six controls (pre-transplant biopsies). All expression data were normalized to two different housekeeper genes (GAPDH and 18S rRNA) and yielded comparable results. Consistent with the immunohistological data CXCR3 mRNA expression as well as expression of the corresponding ligands was found in the tubulointerstitial compartment in all three disease entities (Figure 7). The strong variation of the mRNA data might be explained in part by the differences in disease severity and by the immunohistochgemical observation that accumulation of...
CXCR3-positive cells was commonly focal but the tissue used for mRNA analysis comprised less than 10% of the biopsy. This might also explain the absence of a significant correlation between gene expression and serum creatinine. Nonetheless, a significant correlation was found between the interstitial expression of CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC and the expression of the corresponding receptor CXCR3 (Figure 7, E–G). The best correlation was found for CXCL10/IP-10. Taken together the mRNA data confirm the immunohistological observations of CXCR3 expression predominantly in the tubulointerstitial compartment from biopsies with different forms of glomerulonephritis.

Expression of the Chemokine Receptors CXCR3 and CCR5 Correlates with Morphological and Clinical Features of Progressive Disease

The severity of interstitial involvement correlates well with renal function in various renal diseases.\textsuperscript{35} It is now generally accepted that the tubulointerstitial space plays a significant role in renal disease progression.\textsuperscript{36} As this may relate to infiltrating inflammatory cells their characterization appears of interest. The mean numbers of CXCR3 and CCR5-positive cells rose significantly with the interstitial area involved in the chronic injury process (Figure 8). As described for the distribution pattern, the mean number of interstitial CXCR3-positive cells was higher, with a stronger increase with disease severity as compared to CCR5. A significant numerical correlation was found for CD3-positive T cells and serum creatinine, and both for CXCR3 and CCR5 with serum creatinine and BUN, as clinical markers of renal function (Table 3). The best correlations were found for CXCR3. These data imply that the chemokine receptors CXCR3 and CCR5 are expressed by a significant number of the interstitial infiltrates, with CXCR3-positive cells demonstrating an inverse correlation with renal function. Proteinuria and the percentage of globally sclerosed glomeruli, two indicators of a poor prognosis in glomerular diseases, correlated significantly with the number of CD3-positive T cells, CXCR3, and CCR5-positive cells.

The morphological distribution, and the double-labeling studies demonstrated that interstitial T cells were the main source of CXCR3 expression. Furthermore, a significant numerical correlation between CD3-positive T cells, CCR5, and CXCR3-positive cells was detected (Table 3). In combination with the morphological data, this is consistent with a proportion of the interstitial T cells expressing both receptors.

Discussion

A considerable body of data from in vitro and in vivo studies imply an important role of chemokines and their corresponding receptors during renal inflammation (reviewed in\textsuperscript{5,6}). Besides increasing our understanding of disease mechanisms recent studies in the field focused on two clinically important questions. Can blockade of chemokines modulate disease activity? Which chemokines and receptors might be the most promising therapeutic targets in human kidney diseases?

In this study we describe CXCR3 as a potential diagnostic and therapeutic target in progressive glomerular diseases, ie, IgA nephropathy, lupus nephritis, and membranoproliferative glomerulonephritis (MPGN). Irrespective of the type of glomerular disease, the following main findings were observed: CXCR3-positive cells are relatively rare in glomerular tufts as no evidence for the presence of the classical CXCR3 on mesangial cells could be obtained; the main site of CXCR3 expression was infiltrating T cells in the tubulointerstitium; and there is a correlation between the number of infiltrating CXCR3-positive cells and the degree of renal dysfunction and indicators of progressive renal disease. This correlation is stronger for CXCR3 as compared to CCR5-positive infiltrating cells.

In contrast to published results, our data do not provide evidence for “classical” CXCR3 expression in glomeruli or on cultured mesangial cells.\textsuperscript{22} This is based on the absence of mRNA in microdissected glomeruli, in cultured HMC under basal or stimulated conditions, and on the absence of immunological detection of CXCR3 protein on fixed biopsies. Consistently using FACS analysis of HMC the antibody 1C6 does not result in a positive signal, whereas the antibody 49801.111 results in a significant shift. Apparently, the antibody 49801.111 recognizes an epitope on frozen biopsy tissue present on smooth muscle cells and activated mesangial cells in adult kidneys.\textsuperscript{22,23} The differences between the two anti-CXCR3 antibodies have already been indicated by previous studies.\textsuperscript{8,22} Qin et al\textsuperscript{8} demonstrated that the percentage of CXCR3-positive infiltrating lymphocytes was
Figure 6. Double-immunofluorescence for CXCR3 (A, B, J, and M, green), and CD3-positive cells (E, G, K, and N, red) on a nephrectomy specimen with obstructed uropathy (A–H), and on a renal biopsy with lupus nephritis (I–N, all original X400). A and E: Interstitial CD3-positive cells (E), with a corresponding picture for CXCR3 (A). B and F: Immunofluorescence for CXCR3 (B) with replacement of anti-CD3 by an isotype-matched rat IgG (F). C and G: Immunofluorescence for CD3 (G) with replacement of 1C6 by an isotype-matched mouse IgG (C). D and H: No cellular staining when both antibodies were replaced by isotype-matched IgGs (D, mouse IgG1, H, rat IgG). Double-immunofluorescence for CXCR3 (J and M, green), and CD3 (K and N, red) on a renal biopsy with lupus nephritis (all original magnification, X400). Overlay of red and green is shown in panels I and L (nuclei are counter-stained in blue). Note the prominent interstitial CD3-positive infiltrate infiltrate (K and N) with a corresponding picture for CXCR3 (arrows in M and N illustrate the same cells).
very high in rheumatoid arthritis, in inflamed vaginal mucosa, and in ulcerative colitis, whereas it was lower in normal lymph nodes. Using 1C6 on frozen sections from these tissues the authors described an absence of CXCR3 staining on smooth muscle cells, endothelium, and fibroblasts, which is consistent with our finding. In contrast, immunoreactivity was described with the antibody 49801.111 in vascular smooth muscle cells both in normal and diseased kidneys. As the clone 49801.111 seems to bind to a molecule that is constitutively expressed on smooth muscle cells, the finding of an increased immunoreactivity on glomerular mesangial cells during glomerulonephritis might not be surprising as mesangial cells up-regulate markers of smooth muscle cells during glomerular inflammation. Apparently the epitope recognized by this antibody does not belong to the classical CXCR3 receptor, as we were unable to detect mRNA for CXCR3 in glomeruli and HMC. Romagnani et al demonstrated that expression of CXCR3 (detected by the monoclonal antibody 49801.111) is cell cycle-dependent in human microvascular endothelial cells, but not in mesangial cells. These results in combination with our cell culture experiments, including HMC in various phases of cell cycle, as well as the immunohistochemical staining make it unlikely that cell cycle dependence had an influence on our negative results. Despite the absence of mRNA for CXCR3 on cultured HMC, we could confirm the observation by Romagnani et al, that cultured HMC respond to CXCR3 ligands, by showing both chemotaxis and cell proliferation to the chemokines CXCL9/Mig and CXCL10/IP-10. It is unlikely that HMC contain the splice variant CXCR3-B, recently described by the same group, as RT-PCR covering CXCR-B sequences were negative for isolated glomeruli and mesangial cells. CXCR3-B was expressed by endothelial cells, but not mesangial cells, and furthermore mediated anti-proliferative activity. However the monoclonal antibody 49801.111 recognized both CXCR3-B and the classical CXCR-A in the study by Lasagni et al, indicating that the monoclonal antibody may bind to an epitope common to both CXCR3-A and CXCR-B, and perhaps other receptor variants as well. CXCR3-B contains 51 additional amino acids in the N-terminus and therefore it might be important that the monoclonal antibody 1C6 was raised against the N-terminus of CXCR3. Soejima et al demonstrated high-affinity binding sites and chemotactic responses to CXCL10/IP-10 in non-small cell lung cancer cell lines. This occurred despite absence of mRNA for CXCR3, and of CXCR3 protein studied by FACS analysis using the monoclonal antibody 1C6. Chemotactic responses to CXCL10/IP-10 were inhibited by pertussis toxin, indicating involvement of receptor coupling to Gαi. Binding of radiolabeled CXCL10/IP-10 could not be competed with the CXCR3 ligand CXCL9/Mig. Furthermore, CXCL4/PF4 did not compete with CXCL10/IP-10 indicating that the effects are not mediated via CXCR3-B. This study in combination with our results imply that there might be other receptors (or receptor variants) than CXCR3 involved in CXCL10/IP-10 signaling.

Functional responses by mesangial cells, in the absence of presently known receptors for the chemokine ligand, have previously been demonstrated in mouse mesangial cells. In this study, mouse mesangial cells did react to the chemokines macrophage inflammatory...
protein-2 (MIP-2) and KC with cell migration and up-regulation of CCL2/MCP-1. This occurred in the absence of CXCR2, the only receptor for these ligands known at present.40 As the functional responses to CXCL10/IP-10 and CXCL9/Mig in HMC occur in the absence of the “classical” CXCR3 or the splice variant CXCR-B, we would conclude that the response is due to either an as yet unknown variant of CXCR3 or is mediated by another as yet undefined chemokine receptor, a question that clearly requires further studies. Practically, these results are very relevant for the interpretation of published data using the different commercially available antibodies.

We consider the most intriguing result of our study the potential role of CXCR3-positive cells in the progression of glomerular diseases. In various forms of human glomerular diseases the amount of mononuclear tubulointerstitial infiltration and fibrosis correlates well with renal function and the prognosis of the disease.35,41,42 Defining cell populations involved in the glomerular and tubulointerstitial injury process in human kidney diseases might therefore be of both prognostic, and therapeutic importance. Irrespective of the type of glomerular disease, ie, IgA nephropathy, lupus nephritis, or MPGN, infiltrating inflammatory cells expressing either CXCR3 or CCR5 were mainly localized in the tubulointerstitium, but not in glomeruli. Data in human glomerular diseases indicating a role for CCR5 and its ligand CCL5/RANTES are already available.40,43,44 Increased expression of the CCR5 ligand CCL5/RANTES has been shown to be associated with disease progression in membranous nephropathy.44 CCL5/RANTES expression has been localized to tubular epithelial cells and interstitial infiltrating cells.34 Furthermore, we previously demonstrated that the number of interstitial CCR5-positive cells increased with worsening renal function in various forms of glomerular diseases.20 The current study expands on these findings by showing that the increase of CCR5-positive cells correlates with the severity of interstitial injury. An even higher degree of correlation was noted between CXCR3-positive leukocyte infiltration and the severity of the interstitial injury, global glomerulosclerosis, proteinuria, and renal dysfunction, as assessed by serum creatinine and BUN at the time of biopsy. It should be noted, however, that the higher percentage of CXCR3-positive CD3-positive T cells as compared to CCR5-positive CD3-positive T cells might simply reflect the higher distribution of CXCR3 as compared to CCR5 on peripheral T cells.8 Nonetheless, the highly significant correlation of the CXCR3-positive cell infiltrate with various parameters of renal dysfunction points toward the CXCR3-positive T cells as important mediators in progression of renal disease. Therefore CXCR3 might be a suitable target for therapeutic interventions in progressive human glomerular diseases. It should be stated here that the correlations demonstrated in this study do not establish a causative role of these cells during disease progression. The clinical use of CXCR3-positive cells as a diagnostic tool needs to be addressed in a prospective study.

A number of studies described the expression of the ligands for CXCR3 in renal injury.34,45,46 CXCL10/IP-10 expression was increased in renal allografts undergoing rejection, and both CXCL10/IP-10 and CXCL9/Mig were expressed during transplant glomerulopathy.34,46 CXCL10/IP-10 can be expressed by mesangial cells,47 tubular epithelial cells,48 and interstitial cells,48,50 in response to stimulation eg, by proinflammatory cytokines and lipopolysacharide.30,51 Migration of activated blood lymphocytes toward supernatants obtained from cytokine stimulated tubular epithelial cells was antagonized by about 70% through the blockade of either CCR5 or CXCR3.7 High levels of CCL5/RANTES and CXCL10/IP-10 protein in tubular epithelial cell supernatants were demonstrated.7 In renal biopsies with proliferative glomerulonephritis, a strong expression of CXCL10/IP-10 and CXCL9/Mig has been described in glomeruli and in cells infiltrating the interstitium.45 Using real time RT-PCR we found that mRNA for both the chemokine CXCL10/IP-10 and its receptor CXCR3 was predominantly expressed in the tubulointerstitial compartment, whereas expression of CXCR3 in microdissected normal and diseased glomeruli was too low to allow reliable detection.

Table 3. Correlation between Mean Cell Numbers, Clinical and Morphological Parameters

<table>
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<tr>
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<th>CD3</th>
<th>CXCR3</th>
<th>CCR5</th>
</tr>
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<tr>
<td>Age</td>
<td>sr  = 0.099, p = 0.54 (95%: 0.02 to 0.40)</td>
<td>sr  = 0.24, p = 0.11 (95%: -0.07 to 0.51)</td>
<td>sr  = -0.00, p = 0.99 (95%: -0.32 to 0.32)</td>
</tr>
<tr>
<td>% Globally sclerosed</td>
<td>sr  = 0.45, p = 0.0028 (95%: 0.16 to 0.67)</td>
<td>sr  = 0.58, p &lt; 0.0001 (95%: 0.33 to 0.75)</td>
<td>sr  = 0.48, p = 0.0019 (95%: 0.18 to 0.69)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>sr  = 0.44, p = 0.0055 (95%: 0.13 to 0.67)</td>
<td>sr  = 0.74, p &lt; 0.0001 (95%: 0.55 to 0.85)</td>
<td>sr  = 0.65, p &lt; 0.0001 (95%: 0.42 to 0.8)</td>
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<tr>
<td>BUN</td>
<td>sr  = 0.21, p = 0.211 (95%: 0.13 to 0.51)</td>
<td>sr  = 0.40, p = 0.012 (95%: 0.08 to 0.65)</td>
<td>sr  = 0.36, p = 0.037 (95%: 0.00 to 0.64)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>sr  = 0.38, p = 0.024 (95%: 0.04 to 0.64)</td>
<td>sr  = 0.48, p = 0.0026 (95%: 0.17 to 0.70)</td>
<td>sr  = 0.37, p = 0.033 (95%: 0.02 to 0.63)</td>
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sr, Spearman correlation coefficient, nonparametric correlation; 95%, confidence interval; % globally sclerosed, percentage of globally sclerosed glomeruli; % chronic TI damage, percentage involved in chronic tubulointerstitial injury.
The absence of mRNA for CXCR3 in glomeruli from control kidneys is consistent with our inability to find CXCR3 by either immunohistological or molecular biology methods in HMC. In the tubulointerstitial compartment a good correlation was found between the mRNA expression of CXCR3 and the corresponding ligands. Attempts to establish immunohistochemistry for the chemokines CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC using commercially available antisera did not result in a reliable staining of fixed material in our hands. Therefore the morphological distribution of the CXCR3 ligands in human biopsies needs to be defined in further studies.

The impact of a therapeutic intervention targeting CXCR3 or its ligands has been demonstrated in various animal models. For example, in an adjuvant-induced peritonitis model in the mouse the recruitment of Th1 cells has been demonstrated to be dependent on CXCR3, as anti-mCXCR3 antibodies profoundly inhibited recruitment to the peritoneum.\(^5\) Neutralization of CXCL10/IP-10 was of therapeutic benefit in acute colitis in mice.\(^5\) CXCR3-deficient mice are significantly protected from rejection in the heart transplantation model, and these mice accept allografts with sub-therapeutic doses of cyclosporin A.\(^\text{54}\) Furthermore, anti-CXCR3 antibodies significantly prolonged allograft survival in wild-type animals.\(^\text{54}\) Recently, a nonpeptide chemokine antagonist blocking both CCR5 and CXCR3 has been described (TAK-779), which might be an important new tool for therapeutic intervention potentially blocking two populations important for the progression of human kidney diseases.\(^5\)

Interstitial CD3-positive T cells seem to be the main population expressing both CXCR3 and CCR5.\(^\text{20}\) An overlapping distribution pattern in addition to a significant numerical correlation demonstrates a population of double-positive T cells infiltrating the interstitium in glomerular diseases. Both CXCR3 and CCR5 have been shown to be preferentially expressed by Th1 cells in vitro.\(^\text{10,56}\) The expression of these two receptors by cells infiltrating the tubulointerstitium, and the correlation with disease progression indicate that the progressive interstitial injury process seen in glomerulonephritis might be Th1-driven. The importance of both receptors in allograft rejection is consistent with this view. For example, in cardiac allograft rejection the expression of CXCR3 and CCR5 correlates with the presence and grade of allograft rejection.\(^\text{15}\)

The role of interstitial T cells during progression of glomerular diseases is incompletely understood. The main cell types forming interstitial infiltrates during glomerular diseases are T cells and monocyte/macrophages, and the best correlation between interstitial infiltrates and excretory renal function was found for T cells.\(^\text{57}\) The similar patterns of T cells, and T cell-related chemokine receptors in different progressive glomerular diseases, argues for a common pathway of interstitial injury during disease progression.\(^\text{57,58}\) Glomerular diseases seem to produce a proinflammatory interstitial milieu, which may favor T cell influx and potentially T cell survival. The recruitment of T cells might be mediated through the release of chemokines by activated or injured tubular epithelial cells by “spill over” of glomerular cytokines into peritubular capillaries or other mechanisms causing local injury.\(^\text{5}\) CXCR3-positive T cells mainly represent activated/memory cells. This results in the important question how T cells are activated in the absence of autoantigen during progressive renal disease.\(^\text{8}\) The possibility has to be considered that during tubulointerstitial injury hidden antigens become unmasked serving as neo-antigens. Furthermore, there is some evidence for an antigen-independent pathway of T cell activation through oxygen free radicals and chemokines (\(^\text{16}\) reviewed in\(^\text{56}\)). T cells can be activated by CCL5/RANTES in vitro in an antigen-independent way to proliferate, to up-regulate IL-2 receptor, and express cytokines (eg, IL-2 and IL-5).\(^\text{59}\) These activated T cells might interact with macrophages leading to the production of tumor necrosis factor-\(\alpha\) and IL-1, which might promote a proinflammatory cycle.\(^\text{61}\) Furthermore T cells could be retained in the interstitium through the interaction with fibroblasts, and fibroblasts might promote T cell survival via inhibition of T cell apoptosis.\(^\text{61,62}\) Whatever the potential mechanism previous data and our current study argue for a role of T cells during progressive renal injury.

In conclusion, CXCR3 and CCR5 are expressed by a significant part of interstitial infiltrating T cells during glomerular diseases, and their number correlates with histological and functional disease parameters. The immunohistological data could be confirmed by mRNA data on microdissected renal compartments. Interestingly these findings appear to be independent of the initial glomerular disease, ie, IgA nephropathy, lupus nephritis, or MPGN, and point toward a common pathophysiological pathway for tubulointerstitial injury in progressive glomerulonephritides. The correlations between clinical progression markers and CXCR3-positive cell infiltrate imply that CXCR3 might be a suitable therapeutic target for progressive human glomerular diseases.

Acknowledgments

We thank Sabine Plattner, Anita Joza, Dan Draganovic, Claudia Schmidt, Karin Frach, and Sandra Irrgang for their expert technical assistance.

References


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647
10. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: Flexible programs


15. Fahmy NM, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy

14. Garlet GP, Martins W, Ferreira BR, Milanezi CM, Silva JS: Patterns of

12. Yuan YH, ten Hove T, The FO, Slors JF, van Deventer SJ, te Velde AA:

11. Teleshova N, Pashenkov M, Huang YM, Soderstrom M, Kivisakk P,


23. Grone HJ, Cohen CD, Grone E, Schmidt C, Kretzler M, Schlondorff D,


20. Segerer S, Mack M, Regele H, Kerjaschki D, Schlondorff D: Expres-


18. Melter M, Exeni A, Rettenmayr W, Starling RC, Kivisakk P, Reimann J,


15. Fahmy NM, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy

14. Garlet GP, Martins W, Ferreira BR, Milanezi CM, Silva JS: Patterns of

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18. Melter M, Exeni A, Rettenmayr W, Starling RC, Kivisakk P, Reimann J,


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14. Garlet GP, Martins W, Ferreira BR, Milanezi CM, Silva JS: Patterns of

12. Yuan YH, ten Hove T, The FO, Slors JF, van Deventer SJ, te Velde AA:

11. Teleshova N, Pashenkov M, Huang YM, Soderstrom M, Kivisakk P,


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14. Garlet GP, Martins W, Ferreira BR, Milanezi CM, Silva JS: Patterns of

12. Yuan YH, ten Hove T, The FO, Slors JF, van Deventer SJ, te Velde AA:

11. Teleshova N, Pashenkov M, Huang YM, Soderstrom M, Kivisakk P,


