Expression of the chemokine receptor CXCR1 in human glomerular diseases

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Leukocyte infiltration, a hallmark of renal diseases, is orchestrated in part by the actions of chemokines. The chemokine CXCL8/interleukin (IL)-8 is expressed during renal diseases and allograft rejection, whereas the corresponding receptor CXCR1 has not been described previously. Expression of CXCR1 was characterized in peripheral blood using multicolor fluorescence-activated cell sorter analysis (FACS). CXCR1 was localized in 81 formalin-fixed, paraffin-embedded renal specimens by immunohistochemistry using a monoclonal antibody against human CXCR1. Included were biopsies with crescentic glomerulonephritis (CGN, $n = 22$), immunoglobulin (Ig) A nephropathy ($n = 15$), membranoproliferative glomerulonephritis (MPGN, $n = 17$), lupus nephritis ($n = 12$), membranous nephropathy ($n = 11$), and non-involved parts of tumor nephrectomies ($n = 4$). Consecutive tissue sections of human tonsils, allograft explants, and renal biopsies were stained for CD15- and CD68-positive cells. Expression of CXCR1 and CXCL8/IL-8 mRNA was quantified by real-time reverse transcriptase-polymerse chain reaction of microdissected renal biopsies ($n = 35$) of the same disease entities. By FACS CXCR1 expression was found on polymorphonuclear CXCR1 expression by polymorphonuclear leukocytes (PMNs), natural killer cells, and a subpopulation of monocytes. By immunohistochemistry, CXCR1 expression was found on infiltrating inflammatory cells (predominantly PMNs), as well as on intrinsic renal cells (arterial smooth muscle cells, endothelial cells of peritubular capillaries). The distribution pattern of CXCR1 differed between disease entities. The highest numbers of glomerular CXCR1-positive cells were present in biopsies with MPGN, followed by lupus nephritis, and CGN. CXCR1 might be involved in the recruitment of PMNs to the glomerular tuft, which could be targeted by CXCR1-blocking agents.

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The infiltration of glomeruli and other renal compartments by inflammatory cells is a hallmark of glomerulonephritis (GN), and correlates with renal function and prognosis.[1] Chemokines are members of a family of chemotactic cytokines that play central roles in the recruitment of specific subsets of leukocytes to sites of inflammation or specific microenvironments.[2–6] CXCL8/IL-8 binds to and signals through the chemokine receptors CXCR1 and CXCR2. In addition, CXCL8/IL-8 binds to the Duffy antigen/receptor for chemokines, a promiscuous chemokine-binding protein expressed on peritubular capillaries in inflamed kidneys.[7–9]

CXCL8/IL-8 was the first leukocyte selective chemokine to be identified and its corresponding receptors were the first chemokine receptors to be defined.[7,10,11] *In vitro* studies demonstrated that mesangial cells, podocytes, tubular epithelial cells, and fibroblasts could be potential sources for CXCL8/IL-8.[6,12,13] The expression of CXCL8/IL-8 by intrinsic renal cells is induced *in vitro* by proinflammatory cytokines and growth factors that are upregulated in glomerular diseases.[6,14–16] *In vivo* treatment with an CXCL8/IL-8-blocking antibody reduced glomerular neutrophil accumulation and proteinuria in an acute immune-complex GN in rabbits.[17,18] Induction of CXCL8/IL-8 has also been described in a model of bilateral renal ischemia.[19] In human glomerular diseases, urinary excretion of CXCL8/IL-8 was found to be increased in patients with IgA nephropathy, acute GN, lupus nephritis, MPGN, and cryoglobulinemia.[20] CXCL8/IL-8 mRNA expression was localized to the glomerular tuft and parietal epithelial cells, as well as to tubular epithelial cells, smooth muscle cells, endothelial cells, and some interstitial cells in normal human kidneys, but was found to be reduced during GN.[21]

In contrast to the data on CXCL8/IL-8, the expression of the corresponding receptors CXCR1 and 2, and the cells attracted by CXCL8/IL-8 during renal diseases has not been addressed. Cells which might be responsive to CXCL8/IL-8 include polymorphonuclear leukocytes (PMNs), natural killer cells, a subset of T cells (CD8-positive with effector

function, CD4-positive T cells in TH2 responses), dendritic cells, mast cells, endothelial cells, and chondrocytes, all of which have been shown to express CXCR1.[7,22–31] Expression of CXCR1 has also been described on podocytes *in vitro* and in biopsy samples of membranous GN.[32] The aim of the current study was to characterize the distribution of CXCR1 in glomerular diseases, and to identify diseases with a high number of CXCR1-positive inflammatory cells as potential target diseases for therapeutic intervention using CXCR1 antagonists.

RESULTS

Establishment of the antibody against CXCR1 for the use in immunohistochemistry

A commercial, monoclonal antibody against CXCR1 was extensively tested for immunohistochemistry on formalin-fixed, paraffin-embedded material. Antigen retrieval by autoclaving resulted in a reproducible staining pattern on human tonsils, whereas isotype controls were negative (Figure 1a and c). In the tonsil, CXCR1 was found to be expressed by PMNs, identified by the nuclear morphology (Figure 1i), parallel staining with CD15 (Figure 1c and d), and double immunofluorescence (Figure 1g and h). An additional cell population expressing CXCR1 was found predominantly in the center of follicles (Figure 1e). These cells are not CD3 positive (by double fluorescence) and do not match the pattern of the CD68-positive population on consecutive sections.

All neutrophils, present in the whole blood population, stained strongly for CXCR1 (mean fluorescence intensity of 450). In total, 50% of $CD14^+$ cells were CXCR1 positive. The majority of natural killer cells, characterized as $CD56^{dim}$/$CD16^{high}$ expressed CXCR1, while the $CD16^{neg/dim}$ natural killer cells were negative. B cells ($CD19^+$) and $CD3^+$ T cells were negative for CXCR1.

Cells isolated from tonsil had very few (0.1%) CXCR1-positive cells. Most likely this is because of the isolation procedure which apparently did not retrieve the myeloid subset (2.9%, $CD14^+$, $CD13^+$, $CD68^+$, or $CD209^+$). Consistently with the pattern found in peripheral blood, B cells (73%, $CD19^+$ or $CD20^+$) and T cells (25%, $CD3^+$) of tonsil were CXCXR1 negative. The natural killer cell population present in tonsil (0.8%) was of the $CD16^{neg/dim}$ subtype and in accordance with the observation in peripheral blood was also CXCR1 negative. The population of CXCR1-positive intrafollicular cells found by immunohistochemistry therefore remains poorly defined. The distribution would be consistent with a B-cell population, but this was not confirmed by flow cytometry. Some cells might represent a subset of $CD209^+$ dendritic cells which are found in the tonsil follicles.[33,34] Besides inflammatory cells, CXCR1 expression was prominent on smooth muscle cells of arteries, whereas expression on endothelium of tonsils was not detectable (Figure 1c and h).

In renal biopsies, a good morphological and significant numerical correlation was found between CXCR1- and CD15-positive cells in glomeruli ($P<0.0001$). No morphological correlation was found between the pattern of CD68-positive monocytes/macrophages and CXCR1-positive cells, also based on the relatively high numbers of CD68-positive cells, a small population positive for CXCR1 could not be excluded. The number of both glomerular and interstitial CD68-positive cells greatly outnumbered the CXCR1- and CD15-positive cells.

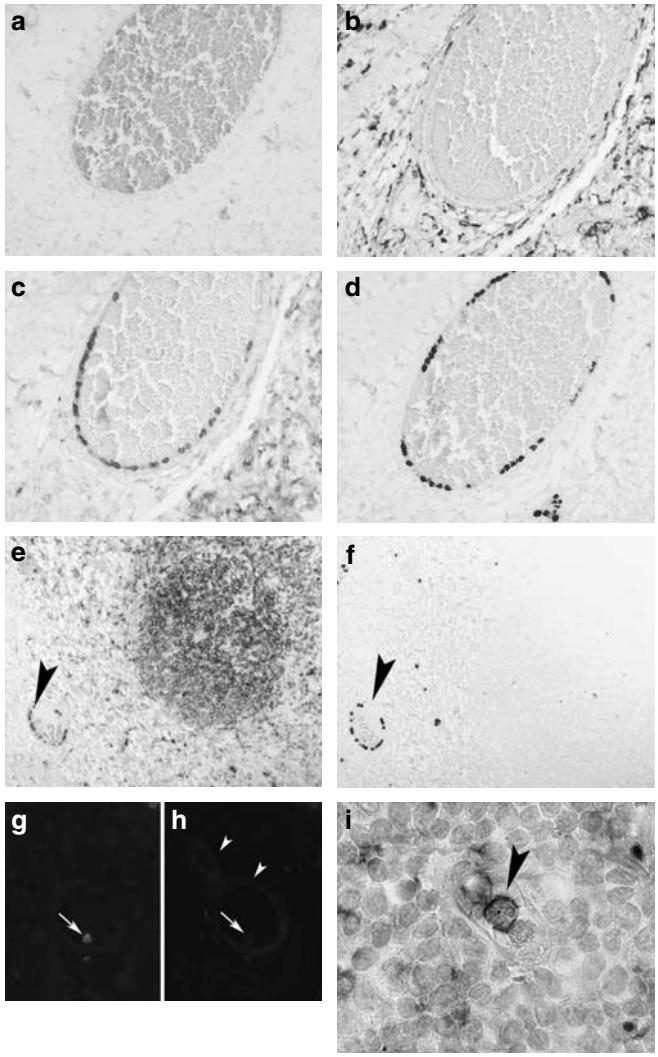

Figure 1 | CXCR1 expression in human tonsils. Immunohistochemistry on consecutive sections of human tonsils, using isotype (**a**) IgG, (**b**) anti-CD68, (**c**, **e**, **h**, **i**) anti-CXCR1, and (**d**, **f**) anti-CD15 monoclonal antibodies (**a–d**, original magnification $\times$ 100; **e**, **f**, original magnification $\times$ 200; **g**, **h**, original magnification $\times$ 400; **i**, original magnification $\times$ 1000). No color product is detectable in the IgG control (**a**). Note the positive cells in the periphery of a large vessel, with similar patterns for (**c**) CXCR1- and (**d**) CD15-positive PMNs, but not (**b**) CD68-positive macrophages. In addition to (arrow in **e**, **f**) CD15-positive cells, (**e**) CXCR1 is expressed by cells within follicles. At a high magnification (**i**, original magnification $\times$ 1000), some positive cells can be clearly identified as PMNs, according to the nuclear structure (arrowhead). Double-immunofluorescence for (**g**) CD15 and (**h**) CXCR1 demonstrates a double positive PMN (arrow) in an artery with CXCR1-positive smooth muscle cells (arrowhead).

Expression of CXCR1 in normal renal tissue

Areas not involved by the tumor from four nephrectomies were used to characterize the expression of CXCR1 in 'normal' renal tissue (Figure 2). Expression of CXCR1 was found on only a small number of cells in the control kidneys (both on intrinsic renal cells as well as on infiltrating leukocytes). Scattered, circulating CXCR1-positive cells were found in glomerular or peritubular capillaries (Figure 2a and b). A prominent expression of CXCR1 was present on smooth muscle cells of small and medium-sized arteries in all four specimens (Figure 2c and d). Positive vessels (5–18) were found in the 20 high-power fields evaluated. The number of positive vessels seems to be higher as compared to biopsies from diseased kidneys (Table 1). CXCR1 expression was found on endothelial cells of a small number of peritubular capillaries (Figure 2e). On rare occasions, positive staining of cells of Bowman's capsule (Figure 2f) or glomerular epithelial cells was detectable (Figure 2g). Tubular epithelial cells were CXCR1 negative.

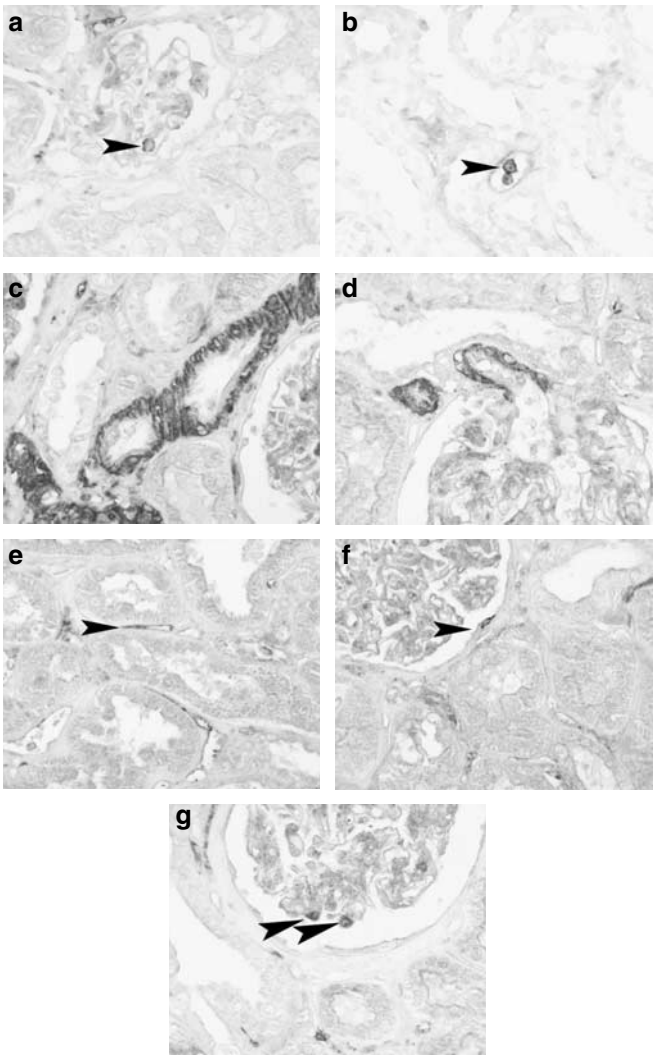

Figure 2 | CXCR1 expression in well preserved renal tissue. Immunohistochemistry on sections of tumor nephrectomies using the monoclonal anti-CXCR1 antibody (**a**, **b**, original magnification $\times 200$; **c**–**g**, original magnification $\times 400$). Please note the low number of CXCR1-positive circulating cells in (**a**) glomerular capillaries or (**b**) peritubular capillaries (arrowhead). A high number of CXCR1-positive smooth muscle cells was present in (**c**, **d**) arteries and arterioles. (**e**, arrowhead) Endothelial cells of a low number of peritubular capillaries were positive for CXCR1. (**f**, arrowhead) Only occasionally were cells of Bowman's capsule or (**g**, arrowhead) glomerular epithelial cells positive for CXCR1

CXCR1 expression in MPGN

Included were 17 biopsies with a membranoproliferative pattern of glomerular injury. These biopsies contained the highest numbers of CXCR1-positive cells within glomeruli (Figures 3 and 4a–c). This is consistent with the observation that it was the disease that showed the highest number of glomerular CD15-positive cells (Table 1). Most of the glomerular cells were PMNs according to the morphology of the nuclei and the distribution (Figure 4c and d). CXCR1-positive cells were sometimes accentuated in the periphery of the glomerular tuft (Figure 4a and c). Of the 17 biopsies, 13 demonstrated a strong glomerular infiltration by CXCR1-positive cells. Additionally, these biopsies demonstrated a conspicuous interstitial accumulation of CXCR1-positive cells (Figure 4b).

In contrast, the number of CXCR1-positive vessels was very low. Only five biopsies contained positive vessels, with only 1–2 positive vessels per 20 high-power fields (Table 1).

CXCR1 expression in CGN

Twenty-two biopsies with crescentic glomerulonephritis (CGN) were included in the study. The number of CXCR1-positive infiltrating cells was high within both glomeruli and the tubulointerstitium (Figure 5a, b and e). CXCR1-positive cells were found to be accumulated within the remaining glomerular tuft and were rarely present within extracapillary crescents (Figure 5a and b). Some biopsies demonstrated a prominent accumulation of CXCR1-positive cells within the interstitium, predominantly in peritubular capillaries (Figure 5e and f). In nine biopsies CXCR1 expression was predominantly found by infiltrating cells in the interstitium, 13 demonstrated expression primarily in glomeruli or in both the interstitium and glomeruli. Only six of the 22 biopsies demonstrated staining of arterial smooth muscle cells and the numbers of positive vessels (between 1 and 3 per 20 high-power fields) were low. A good morphological correlation was found between interstitial CXCR1-positive infiltrating cells and CD15- positive cells (Figure 5e and f). CXCR1-positive cells were greatly outnumbered by CD68-positive cells in glomeruli and the tubulointerstitium (Figure 5c and d).

CXCR1 expression in lupus nephritis

Twelve biopsies from patients with lupus nephritis (three type III, nine type IV) were examined. The overall number of CXCR1-positive infiltrating cells was high and similar to what was seen in biopsies with CGN (Figures 5 and 6). CXCR1-positive inflammatory cells were common in (Figure 6c) and around the glomeruli (Figure 6a and b). Most of the

Table 1 | Mean number of CXCR1-positive cells or vessels in the different disease entities

Disease	Mean number of CXCR1 positive interstitial cells	Mean number of CXCR1 positive cells per glomerulus	Mean number of CXCR1 positive vessels	Mean number of CD15 positive interstitial cells	Mean number of CD15 positive cells per glomerulus	Mean number of CD68 positive cells per glomerulus
Membr N	0.51±0.1	0.42±0.1	1.82±0.77	2.14±1.07	0.6±0.26	2.34±0.37
IgA N	0.7±0.16	0.83±0.23	2.33±0.30	1.19±0.25	0.84±0.37	3.93±0.87
Lupus N	1.4±0.49	1.36±0.26	3.17±1.67	1.13±0.22	2.3±0.63	8.26±1.48
CGN	1.17±0.15	1.25±0.4	0.41±0.42	4.8±0.74	3.3±0.88	5.61±0.91
MPGN	1.4±0.51	2.45±0.43	0.47±0.21	2.15±0.56	5.5±0.96	10.63±1.84

CGN, crescentic GN; IgA N, IgA nephropathy; Lupus N, lupus nephritis; Membr N, membranous nephropathy; MPGN, membranoproliferative GN.

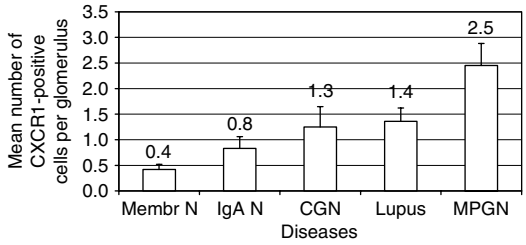

Figure 3 | Mean number CXCR1-positive glomerular cells in the studied disease entities. MPGN vs Membr N, $P<0.01$; Membr N, membranous nephropathy; IgA N, IgA nephropathy; CGN, crescentic GN; Lupus, lupus nephritis; MPGN, membranoproliferative GN.

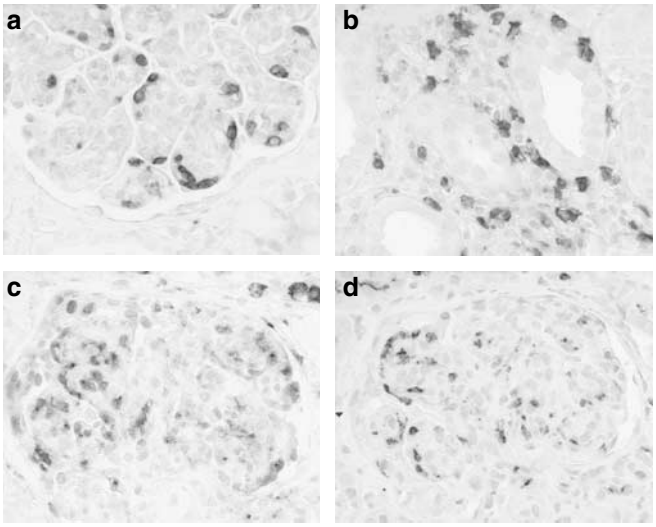

Figure 4 | CXCR1 expression in MPGN. Immunohistochemistry on sections of biopsies with (**a**–**d**) MPGN using the (**a**, **b**, **c**) monoclonal anti-CXCR1 antibody or (**d**, original magnification ×400) anti-CD15. (**a**, **c**) MPGN is the disease entity with the highest number of CXCR1-positive infiltrating cells within glomeruli. (**b**) Illustrates the high number of CXCR1-positive cells within the tubulointerstitium. (**c** and **d**) Consecutive sections of a glomerulus illustrating the corresponding patterns of CD15- and CXCR1-positive cells.

samples demonstrated either a mixed pattern of staining or with a principle expression by infiltrating cells within the glomeruli. Smooth muscle cells of arterioles were commonly positive for CXCR1 (Figure 6a). In half of the biopsies, vascular smooth muscle cell expression of CXCR1 was

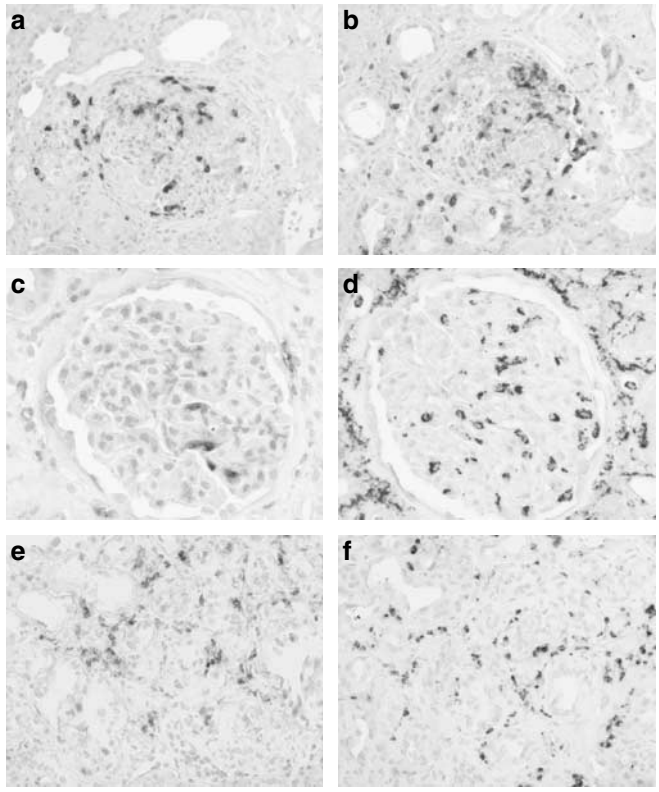

Figure 5 | CXCR1 expression in CGN. Immunohistochemistry on sections of biopsies with crescentic GN using the (**a**–**c**, **e**) monoclonal anti CXCR1, (**d**) anti-CD68, and (**f**) anti-CD15 (**a**, **b**, **e**, **f**, original magnification ×200; **c**, **d**, original magnification ×400). (**a** and **b**) Illustrate the high number of CXCR1-positive cells in glomeruli. (**c** and **d**) Demonstrate that the pattern of (**c**) CXCR1 is different to the distribution of (**d**) CD68-positive monocytes/macrophages (on consecutive sections). Please note the low number of CXCR1-positive cells in this case, outnumbered by CD68-positive cells. Monocytes/macrophages demonstrate a different pattern, particularly within the tubulointerstitium. (**e** and **f**) Illustrate the similar distribution pattern of interstitial (**e**) CXCR1- and (**f**) CD15-positive infiltrating cells.

detectable, with a range between one and 20 positive vessels per 20 high-power fields (Table 1).

CXCR1 expression in IgA nephropathy

According to the subclassification of IgA nephropathy by Haas,[35] one biopsy was subclass II, five were subclass III, and nine biopsies were of subclass IV. In these biopsies, overall

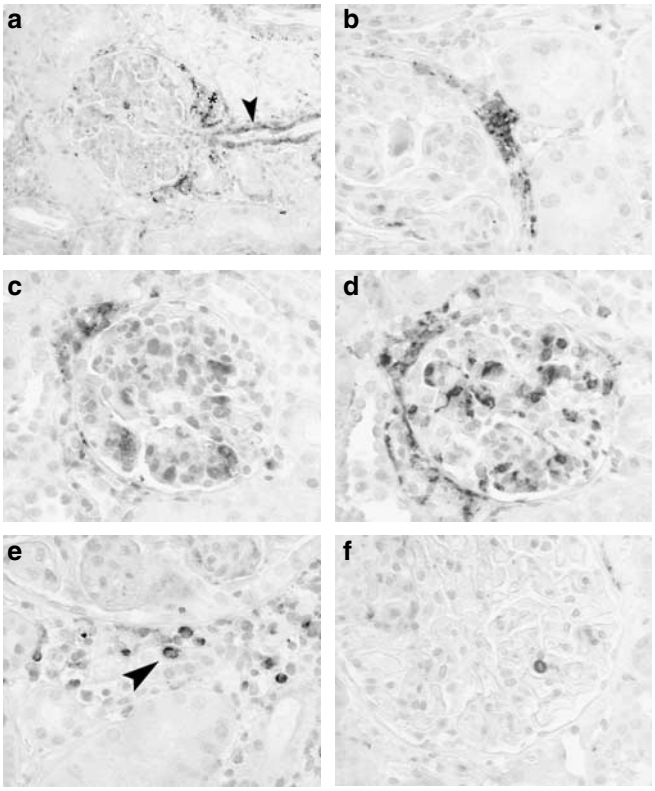

Figure 6 | CXCR1 expression in lupus nephritis and membranous nephropathy. Immunohistochemistry on sections of biopsies with (**a**–**e**) lupus nephritis and with (**f**) membranous nephropathy using the (**a–c**, **e**, **f**) monoclonal anti-CXCR1 antibody, or a (**d**; **a** original magnification ×200) monoclonal anti-CD68 antibody (**b–f** original magnification ×400). (**a**) Illustrates the CXCR1-positive periglomerular infiltrate (star) and positive smooth muscle cells (arrowhead, compare with **b**). (**c** and **d**) Consecutive sections of the same glomerulus which illustrates that CD68-positive monocytes/macrophages outnumber CXCR1-positive cells. (**e**) A prominent CXCR1-positive interstitial infiltrate is illustrated (some CXCR1-positive cells infiltrate the tubular epithelium, arrowhead). (**f**) CXCR1-positive cells were only occasionally seen in membranous nephropathy.

number of CXCR1-positive infiltrating cells was low (Figure 3). Scattered positive cells were found in glomerular and peritubular capillaries. Six cases demonstrated arterial smooth muscle staining (with 1–13 positive vessels per 20 high-power fields). The number of CXCR1-positive cells did not differ between subclass III and subclass IV (according to the classification by Haas M[35]).

Expression of CXCR1 in membranous GN

All 11 biopsies with a membranous nephropathy contained a low number of CXCR1-positive cells, similar to that seen in biopsies with IgA nephropathy (Figure 3). Four biopsies demonstrated 4–6 positive vessels per 20 high-power fields.

CXCL8/IL-8 and CXCR1 mRNA expression in renal biopsies

To further study the mRNA expression of CXCL8/IL8 and CXCR1, we performed real time reverse transcriptase-polymerse chain reaction on microdissected renal biopsies.

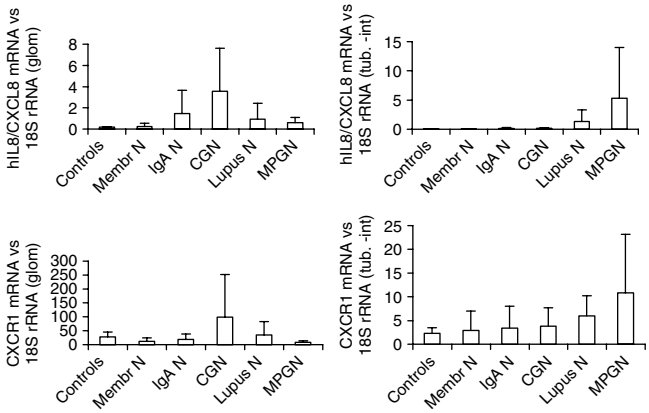

Figure 7 | CXCL8/IL-8 (upper panel) and CXCR1 (lower panel) mRNA expression in renal biopsies microdissected into glomeruli (left) and the tubulo-interstitial (right) compartment (Membr N: membranous nephropathy, IgA N: IgA nephropathy, CGN: crescentic GN, Lupus: lupus nephritis, MPGN: membrano-proliferative GN).

Expression was normalized to 18S ribosomal RNA. The amount of CXCL8/IL-8 mRNA demonstrated a strong variation between the groups tested (Figure 7). The most prominent expression of CXCL8/IL-8 in the glomerular compartment was found in biopsies with CGN, IgA nephropathy, and lupus nephritis (Figure 7, upper left). Consistently, CGN was the disease with the strongest expression of CXCR1 within glomeruli (Figure 7, lower left). Within the tubulo-interstitial compartment, MPGN and lupus nephritis demonstrated the highest expression of both the ligand CXCL8/IL-8 as well as the corresponding receptor CXCR1.

DISCUSSION

Glomerular accumulation of CXCR1-positive infiltrating cells was most prominent in three diseases, that is, MPGN, CGN, and lupus nephritis. Both the distribution of CD15-positive cells on serial sections and the nuclear morphology of CXCR1-positive cells was consistent with PMNs. We also demonstrated the expression of CXCL8/IL-8 and CXCR1 mRNA in glomeruli with CGN and lupus nephritis. Excretion of CXCL8/IL-8 has been described in the urine of patients with acute GN, lupus nephritis, and MPGN.[20]

Unexpectedly, MPGN was found to be the disease with the highest numbers of CXCR1-positive infiltrating cells within glomeruli. Although accumulation of PMNs in MPGN has been described ('exudative phase'), the potential role of these cells in this disease is largely unexplored.[36] MPGN is a morphological entity characterized by increased glomerular cellularity (via proliferation of intrinsic cells and recruitment of leukocytes), thickening, and double contours of the basement membrane.[37] Accumulation of infiltrating inflammatory cells, particularly in cases associated with cryoglobulins, has been well described, but the majority of these cells have been identified as monocytes/macrophages.[36,38] The authors mention that a 'moderate' infiltration of neutrophils

was commonly found in type I MPGN.[36] Furthermore, an increased number of PMNs has been described in glomeruli in about a third of the cases.[39] It was rather unexpected that 13 out of 17 biopsies with an MPGN pattern of injury demonstrated a strong glomerular accumulation of CXCR1-positive cells. The biopsies were randomly chosen according to the availability of remaining material. Based on experimental studies, neutrophil recruitment is thought to be an early event in the inflammation cascade during GN. In human MPGN, the accumulation of PMNs appears to occur over a longer period as these cells can be detected in randomly chosen renal biopsies. A pathogenetic role of CXCR1-positive PMNs can be easily envisioned as these cells are a rich source for reactive oxygen species, proteases (degrading extracellular matrix including glomerular basement membrane), and cytokines promoting cellular activation and further recruitment of inflammatory cells.[40,41] This is mainly based on animal models and further studies in human MPGN are clearly needed.

A role for CXCL8/IL-8 in lupus nephritis has been suggested by Rovin *et al.*[42] This group identified polymorphisms in the CXCL8/IL-8 promoter region and the IL-8-845C polymorphism was more common in African Americans with severe lupus nephritis.[42] Unfortunately, to the best of our knowledge, the functional consequences of this polymorphism are not known at present. In contrast to the data presented by Wada[20] who described an increased excretion of CXCL8/IL-8 in the urine of patients with lupus nephritis, Rovin *et al.*[43] could not demonstrate increased CXCL8/IL-8 excretion in this patient group.

The role of CXCL8/IL-8 in antineutrophil cytoplasmic autoantibody-associated GN has been addressed by Cockwell *et al.*[44] Expression of CXCL8/IL-8 was described within glomeruli in segmental lesions, crescents and parietal epithelial cells, and the protein colocalized with infiltrating neutrophils.[44] Anti-neutrophil cytoplamic antibodies-stimulated neutrophils released large amounts of CXCL8/IL-8, which prevented neutrophil transmigration in an *in vitro* assay.[44] Cockwell *et al.*[44] found an accumulation of neutrophils within glomerular capillaries as well as in the tubulointerstitium, very similar to the general distribution of CXCR1 staining demonstrated in our current study. Several groups have demonstrated a prominent accumulation of neutrophils in biopsies from patients with CGN, both in glomeruli and the tubulointerstitium consistent with our results.[45,46] In addition, PMNs have been extensively studied as the antigen source in anti-neutrophil cytoplamic antibodies-associated vasculitis, but the role of PMNs as infiltrating cells in CGN has not been widely addressed.[45]

Interestingly, the three entities characterized by the highest numbers of CXCR1-positive cells are diseases which also demonstrate prominent recruitment of monocytes/macrophages to glomeruli. The recruitment of CXCR1-positive PMNs could be an early event, and might promote downstream recruitment of macrophages. Macrophages might also be involved in the clearance of apoptotic PMNs, as described in model systems.[47] On the other hand, in a model of lung injury, another amplification loop has been described in which the presence of monocytes significantly accelerated and increased alveolar neutrophil accumulation.[48] Such a mechanism would explain the finding of CXCR1-positive PMNs in glomeruli of chronic MPGN. Here, we suggest a model in which waves of PMNs are recruited to glomeruli in the course of chronic glomerular diseases (particularly in those with a prominent accumulation of macrophages).

Blocking CXCR1 in glomerular diseases might be a new therapeutic approach in MPGN, lupus nephritis, and CGN. Repertaxin is a small molecule, noncompetitive allosteric inhibitor of CXCR1 and CXCR2.[49] This substance locks CXCR1 in an inactive conformation and prevents signaling. Recruitment of PMNs into the reperfused liver was blocked by repertaxin in a rat model of ischemia–reperfusion injury.[49] Repertaxin has been used in phase I clinical studies and was described as safe and well tolerated.[49] Other CXCR1 antagonists are under investigation.[50]

In addition to inflammatory cells, CXCR1 was also found to be expressed by intrinsic renal cells. By immunohistochemistry in normal human tissue, a strong reactivity on smooth muscle cells of a subset of small and medium-sized arteries was demonstrated. The number of CXCR1-positive arteries was high in normal control tissue, whereas it appeared to be lower during GN. Expression of CXCR1 by smooth muscle cells has not been described in the literature and therefore needs to be confirmed using other methods. In contrast to the positive staining of smooth muscle cells, endothelial cells of arteries demonstrated no CXCR1 expression. Positive staining of endothelial cells was found on a low number of peritubular capillaries. Again this staining was pronounced in normal tissue as compared to biopsies with glomerular diseases. For technical reasons, we cannot exclude a low number of interstitial CXCR1-positive endothelial cells in areas with strong tubulointerstitial inflammation. CXCR1-positive podocytes where rarely seen in normal kidneys and CXCR1 expression by podocytes did not correlate with glomerular injury. CXCR1 expression has been demonstrated in cultured podocytes, and in a case of membranous nephropathy.[32] None of the intrinsic renal cells demonstrated an induction of CXCR1 during inflammatory injury. The ligand CXCL8/IL-8 was clearly detectable on mRNA level and might be involved in a house keeping function of CXCR1.[21] A note of caution should be added as renal biopsies only reflect a snap shot in a complex disease course and the 'well-preserved' tissue from tumor nephrectomies might not represent the 'normal' situation.

In summary, this is the first study that demonstrates the expression of CXCR1 by PMNs both in the glomerular and tubulointerstitial renal compartment in glomerular diseases. The most prominent glomerular accumulation of CXCR1 was found in MPGN, followed by lupus nephritis, and CGN. The pathogenic role of these cells is still incompletely understood, but these cells may represent a novel therapeutic target.

MATERIALS AND METHODS

Renal biopsies

A total of 81 human archival renal biopsies, from patients with CGN ($n=22$), IgA nephropathy ($n=15$), MPGN ($n=17$), lupus nephritis ($n=12$), membranous nephropathy ($n=11$), and normal renal tissue (derived from tumor nephrectomies, $n=4$) were included. Renal biopsies were fixed in 4% phosphate-buffered formalin, embedded in paraffin. and cut at 2 μm. The histomorphologic diagnosis was based on light microscopy, immunohistochemistry, and electron microscopy.

Immunohistochemistry

The protocols for immunohistochemistry have previously been described in detail.[51] The primary antibody was diluted in 10% non-fat dry milk and incubated overnight (anti-human CXCR1 (IL-8 RA), clone 42705.111, R&D Systems, Minneapolis, MN, USA). For the description of the CXCR1-positive cell population, antibodies against CD68-positive monocytes/macrophages (clone PG-M1, DAKO, Glostrup, Denmark), CD3-positive T cells (clone CD3-12, rat anti-human, Serotec, Oxford, UK), CD79-positive B cells (clone JCB 117, Dako, Glostrup, Denmark), CD15-positive cells (clone 80H5, Immunotech, Marseille Frankreich), and smooth muscle antigen (SMA, clone 1A4, Dako, Glostrup, Denmark) were used as described previously.[52]

Immunofluorescence

Double fluorescence was used on human tonsils to further define the CXCR1-expressing cell populations. After the biotinylated secondary antibody slides were exposed to Streptavidin/fluorescein isothiocyanat (FITC) complex (Vector, Burlingame, CA, USA). Consecutively, slides were rinsed, exposed to the rat-anti CD3 Antibody (Serotec) followed by an anti-rat Cy3 antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Double fluorescence for CXCR1 and CD15 was performed with isotype-specific secondary antibodies (anti-mouse IgG2 biotinylated, 1:100, Caltag, Burlingame, CA, USA; anti-mouse IgM FITC conjugated, 1:25; Vector). As negative controls, each antibody was replaced by isotype controls (not illustrated).

Flow cytometry

For whole blood staining, serum was removed by three washing steps in isotonic phosphate-buffered saline. In total, 50 μl of packed whole blood cells or 0.5×10^6 cells of purified blood mononuclear cells or lymphocytes from tonsil were used for staining. Cells were first Fc-receptor-blocked by treatment with 1 μg of human immunoglobulin G (in phosphate-buffered saline with 0.5% bovine serum albumin)/10^5 cells for 15 min at room temperature. Monoclonal antibodies were then directly added to this reaction and incubated for 45 min on ice. Antibody combinations were: phycoerythrin-labeled CXCR1 (Clone 42705, R&D, Minneapolis, MN, USA) combined with FITC-labeled CD3 (UCHT1, BD Pharmingen, San Diego, CA, USA) and allophycocyanin-labeled CD56 (N901/NKH-1, Beckman Coulter, Fullerton, CA, USA) or FITC-labeled CD19 (HIB19, BD Pharmingen), and APC-labeled CD14 (TueK4, Caltag); or APC-CD14 and FITC-CD68 (KP1, Dako). Four marker monoclonal antibody combinations employed: phycoerythrin-labeled CXCR1 and pacific blue (PB)-labeled CD3 (UCHT1, Dako) combined with APC-CD56 and FITC-labeled CD16 (3G8, BD Pharmingen); or APC-CD209 (Clone 120507, R&D) and FITC-CD68; or APC-CD28 (CD28.2, BD Pharmingen) and FITC-CD27 (M-T271, BD Pharmingen). Following the staining procedure, whole blood cells were subjected to a red blood lysis step using a commercially available whole blood lysing kit. Propidium iodide (1 μg/ml; Sigma Aldrich, Munich, Germany) was added to the stained samples immediately before acquisition, which employed either a fluorescence-activated cell sorter Calibur and Cell Quest Pro software (Becton Dickinson, Mountain View, CA) or CyAn-ADP (Dakocytomation, Hamburg, Germany) and Summit 4.1 Software. For analysis, gates were set on PI-negative (viable) cells and on lymphocytes, neutrophils, or myeloid cells as determined by forward and sideward scatter.

Isolation of cells

Peripheral blood mononuclear cells were obtained from heparinized venous blood samples of healthy volunteer donors using Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. The mononuclear cell fraction was collected, washed with phosphate-buffered saline, counted, and cryopreserved.

For isolation of lymphocytes from tonsil, tissue specimens were covered with ice-cold *N*-2-hydroxyethylpiperazine-*N*[prime]-2-ehanesulfonic acid-buffered saline (without Mg^{2+} and Ca^{2+}), minced into small pieces and passed through a fine wire mesh. Larger fragments were allowed to settle before supernatant was collected and passed through a cell strainer of 20 μm (BD Falcon, Bedford, MA, USA). Lymphocytes were counted and cryopreserved in aliquots.

Real time reverse transcriptase-polymerse chain reaction

To quantify the mRNA expression of CXCL8/IL-8 and the corresponding receptor CXCR1, we used reverse transcriptase-polymerse chain reaction as previously described.[52] Included were microsdissected renal biopsies from a multicenter renal biopsy bank (the European renal cDNA bank). RNAs were from tumor nephrectomies ($n=5$), CGN ($n=8$), lupus nephritis ($n=8$), MPGN ($n=4$), membranous nephropathy ($n=6$), and IgA nephropathy ($n=7$).

Real time reverse transcriptase-polymerse chain reaction was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems, Darmstadt, Germany). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. Target gene forward and reverse primers and probes for CXCR1 were designed with Primer Express™ (Applied Biosystems, Darmstadt, Germany): forward primer 5′-CGCCAGGCTTACCATCCA-3′, reverse primer 5′-CAAACAGCGGCACGATGA-3′ and fluorescent-labeled probe 5′-FAM-TCCGCAACACCATCCGCCATT-TAMRA. Commercially available predeveloped TaqMan assay reagents were used for the internal standard 18S ribosomal RNA and the target gene CXCL8/IL-8.

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. All measurements were performed in duplicates. Controls consisting of bidistilled H_2O were negative in all runs.

Evaluation and statistics

Positive cells were counted in up to 15 high-power fields (original magnification $\times 400$, 0.0743 mm^2) by an observer blinded to the diagnosis of the biopsy. The number of glomeruli, and positive cells per glomeruli were counted for CXCR1, CD15, and CD68. Numbers

are given as means and standard error of the mean. The statistical analysis was performed using the InStat® program (Version 3.05 for Windows, Intuitive Software for Science, San Diego, CA, USA). For the comparison of mean numbers, the non-parametric Mann–Whitney *U*-test was used. A $P<0.05$ was considered to be statistically significant.

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