Nephrol Dial Transplant (2007) 22: 1720–1729
doi:10.1093/ndt/gfm007
Advance Access publication 13 February 2007

Original Article

NDT
Nephrology Dialysis Transplantation

Expression of the chemokine receptor CCR1 in human renal allografts

Verena Mayer[1], Kelly L. Hudkins[2], Florian Heller[1], Holger Schmid[1], Matthias Kretzler[1], Ulrike Brandt[1], Hans-Joachim Anders[1], Heinz Regele[3], Peter J. Nelson[1], Charles E. Alpers[2], Detlef Schlöndorff[1] and Stephan Segerer[1]

[1]Medizinische Poliklinik-Innenstadt, University of Munich, Germany, [2]Department of Pathology, University of Washington, Seattle, WA, USA and [3]Clinical Institute of Pathology, University of Vienna, Vienna, Austria

Abstract

Background. Chemokines are involved in the recruitment of leukocytes to vascularized allografts. CCR1 is a receptor for various proinflammatory chemokines and CCR1 blockade reduces renal allograft injury in rabbits. The purpose of the study was to characterize CCR1-positive cells in human renal allografts.

Methods. Formalin-fixed, paraffin-embedded allograft nephrectomies ($n=9$) and non-involved parts of tumour nephrectomies ($n=10$) were studied. Immunohistochemistry for CCR1, CD3 and CD68 was performed on consecutive sections. Double immunofluorescence for CCR1 and CD3, CD20, CD68, DC-SIGN and S100 was used on selected cases. Expression of CCR1 mRNA and the ligands CCL3 and CCL5 was studied in renal allograft biopsies with acute rejection ($n=10$), with chronic allograft nephropathy ($n=8$) and controls ($n=8$).

Results. CCR1 protein was expressed by circulating cells in glomerular and peritubular capillaries, colocalizing with CD68. In renal allografts CCR1-positive cells were present within glomerular tufts, but only scattered CCR1-positive cells were found in tubulointerstitial infiltrates. CCR1 did not colocalize with the majority of CD68-positive cells in the interstitium. The small number of CCR1-positive interstitial cells were identified as CD20- or DC-SIGN-positive by double immunofluorescence. CCR1 mRNA was significantly increased in renal biopsies with acute allograft rejection ($P<0.001$), and with chronic allograft nephropathy ($P<0.05$), it correlated with the expression of CCL3 and CCL5, and with serum-creatinine.

Conclusions. CCR1 mRNA expression was associated with renal function in allografts. CCR1 protein expression was restricted to monocytes, CD20-positive B cells and DC-SIGN-positive dendritic cells. Thus most interstitial macrophages were CCR1 negative, which may relate to down-regulation after migration into the interstitium in human renal allografts.

Keywords: allograft rejection; CCL5; CCR1; CD20; chemokines; DC-SIGN

Introduction

In a complex cascade of interactions between soluble cytokines, chemokines, adhesion molecules and the corresponding receptors, leukocytes are guided towards sites of inflammation [1,2]. In this process, chemokines trigger the firm adhesion of leukocytes via activation of integrins, as well as the directed migration after extravasation [3,4]. The role of chemokine biology in the recruitment of inflammatory cells into the kidney and particularly in renal allografts has been a major area of study [5–7]. Genetic variations in these genes can impact allograft survival, and chemokines and their receptors represent promising therapeutic targets [5,8].

The chemokine receptor CCR1 shares ligands with CCR5 (e.g. CCL5, CCL3) and binds further inflammatory chemokines including CCL7, CCL14-16 and CCL23 [9]. CCR1 is expressed by monocytes/macrophages (~87% of the peripheral blood), natural killer cells, a minor population of T cells, and bone marrow stromal cells and increases in allograft recipients in peripheral blood before rejection [10–12]. The chemokine receptors CCR1 and CCR5 demonstrated a differential impact on macrophage recruitment *in vitro* [11]. Whereas CCR1 mediated firm adhesion, leucocyte spreading was found to be dependent on CCR5 [11].

In models of progressive renal failure (e.g. Alport disease of collagen 4A3-/- mice, lupus nephritis of MRLlpr/lpr mice), a blockade of CCR1 by a small molecule, non-peptide, CCR1 antagonist (BX-471) demonstrated significant therapeutic impact [13]. Treatment with BX-471 led to a reduction in interstitial

Correspondence and offprint requests to: Stephan Segerer, Medizinische Poliklinik-Innenstadt, Pettenkoferstrasse 8a, 80336 Munich, Germany. Email: stephan.segerer@lrz.uni-muenchen.de

infiltrates, and reduced tubular injury and fibrosis [13]. In a heterotopic heart allograft model, treatment with Met-RANTES (a partial CCR1 and CCR5 antagonist) significantly reduced intimal thickening, and markedly decreased the infiltration of T lymphocytes and monocytes/macrophages into transplanted hearts [14]. Met-RANTES also prevented chronic renal allograft injury in rats [14,15] and the CCR1 antagonist BX-471 prolonged survival and preserved renal allograft integrity in a rabbit allograft model [16]. Therefore, CCR1 represents one important chemokine based target in renal allograft rejection.

During human renal allograft rejection and chronic allograft nephropathy, T cells and macrophages infiltrate different renal compartments, e.g. the tubulointerstitium and renal arteries [17]. To date, the distribution of the chemokine receptors CXCR3, CXCR4, CX3CR1 and CCR5 have been characterized in human renal allograft rejection. The receptors are expressed by T cells infiltrating the tubular epithelium (tubulitis, a hallmark of interstitial rejection) and the subendothelial area during vascular rejection [18]. CCR2 and CX3CR1 were found on infiltrating macrophages [18,19]. CCR1 mRNA was found to be expressed in renal allograft nephrectomies [19]. The only study so far, which localized CCR1 in cryosections of human renal allograft biopsies described an increased number of CCR1-positive cells in glomeruli during rejection, correlating with the number of macrophages [20].

The current study was performed to better characterize the CCR1-positive cell populations in renal allografts using real-time RT-PCR, single colour immunohistochemistry and multiple colour immunofluorescence.

Subjects and methods

Study population

Included were consecutive sections from renal allograft nephrectomies ($n = 9$), and non-involved areas of tumour nephrectomies ($n = 10$). A nephrectomy from a patient with severe interstitial inflammation due to obstructive uropathy and human tonsils were used to establish the antibodies as well as single and double-labelling techniques. No clinical information was available for the nephrectomy specimens as the approval of the University of Washington internal review board for human subjects prescribes that no patient identifiers may be linked to studies involving nephrectomy specimens. We were restricted to nephrectomy specimens as the anti-CCR1 antibody used resulted in a reliable staining pattern in nephrectomy tissue but not in biopsies, most likely due to differences in the fixation.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded materials similar to that described previously [21]. Sections were dewaxed in xylene and rehydrated in a graded series of ethanol. Incubation with 3% H_2O_2 blocked endogenous peroxidases and the Avidin/Biotin blocking Kit (Vector, Burlingame, CA) was used to block endogenous biotin. Antigen retrieval was performed in an autoclave oven in antigen retrieval solution (Vector). Incubation with the polyclonal antiserum was performed overnight in 10% non-fat dry milk. The biotinylated secondary antibodies (Vector), and the ABC reagent (Vector) were applied for 30 min each. 3′3′Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black colour product) served as the detection system.

The antihuman CCR1 antibody used is a polyclonal goat serum raised against a C-terminal CCR1 peptide. The antiserum has previously been used in FACS analysis [22,23] and in immunohistochemistry on frozen tissue [24]. As negative controls we used non-immune goat serum and pre-incubation with the peptide used for immunization, which both demonstrated an absence of black colour product.

To characterize the CCR1-positive cell types, antibodies against CD68 (Clone PG-M1, DAKO Germany, Hamburg), CD3 (clone: CD3-12, rat anti-human, Serotec, Oxford, UK) were used on consecutive sections or against CD68, CD20 (clone L26, DakoCytomation, Dako), CD34 (Accurate clone QBEND/10, ACCURATE CHEMICAL & SCIENTIFIC CORPORATION; NY; USA), DC-SIGN (clone DCN46, BD Pharmingen), S-100 (polyclonal rabbit, DAKO Cytomation) in double-labelling immunofluorescence on human tonsils and selected transplant nephrectomies.

Establishment of the CCR1 staining

The polyclonal antiserum against CCR1 resulted in a reliable staining pattern after a heat-based antigen retrieval, and overnight incubation of the slides and a three-step amplification protocol (Figure 1). Negative controls in the form of non-immune goat serum (Figure 1C and D), and pre-absorption of the antibody with the peptide used for immunization resulted in the absence of positive staining signal (Figure 1E and F). In the tonsil a perifollicular cell population was CCR1-positive (Figure 1A), as well as a population of large intrafollicular cells.

Immunofluorescence

Double immunofluorescence for CCR1 and CD3, CD20, CD34, CD68, smooth muscle actin, DC-SIGN and S100 was performed on human tonsils, and selected transplant nephrectomies as previously described [25]. In brief, after antigen retrieval, slides were incubated with the CCR1 antiserum over night. This was followed by an exposure to a biotinylated anti-goat antibody (Santa Cruz) and incubation with FITC-labelled strepavidin. This was followed by another series of incubations using directly labeled secondary antibodies.

Real-time RT-PCR

The real-time RT-PCR experiments were performed as described [26–29] and quantification of the templates was carried out according to the standard curve method. Therefore, serial dilutions of standard cDNA from a

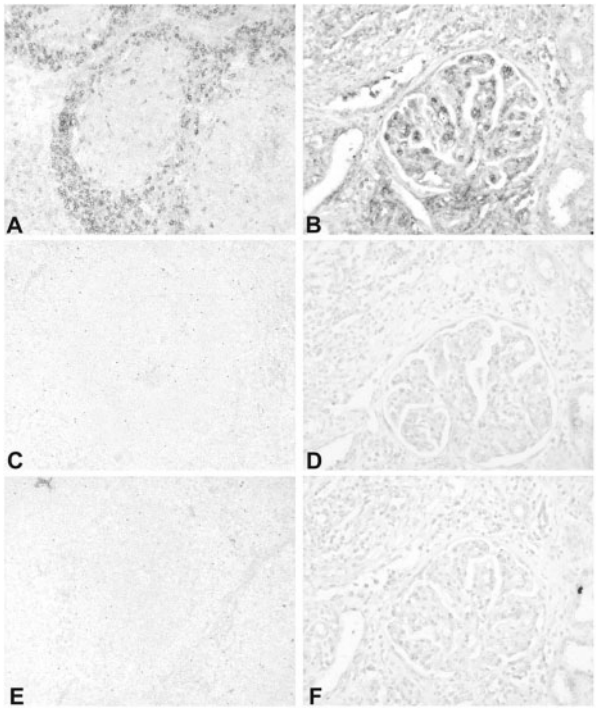

Fig. 1. Immunohistochemistry was performed on sections from human tonsils (**A**, **C**, **E**) and from an allograft nephrectomy (**B**, **D**, **F**) with a polyclonal antiserum against CCR1 (A, B), non-immune goat serum (C, D), and the antiserum against CCR1 preabsorbed with the peptide (E, F, orig. X100 A, C, E, orig. 200 B, D, F).

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 [30]. All measurements were performed in duplicate. Controls consisting of bidistilled H2O were negative in all runs. The renal biopsies were obtained from a multicentre renal biopsy bank (ERCB, the European Renal cDNA Bank). Informed consent was obtained before renal biopsies were performed.

Microdissected tubulointerstitial compartments from allograft biopsies with acute rejection (AR; $n=10$), and chronic allograft nephropathy (CAN; $n=8$) were analysed (Table 1). For control biopsies, renal tubulointerstial tissue was derived from pre-transplantation kidney biopsies (Pre Tx; $n=8$) during cold ischaemia time from living ($n=4$) and cadaveric donors ($n=4$).

Real-time RT-PCR 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). Commercially available pre-developed TaqMan reagents were used for the target gene CCR1, CCL5/RANTES and CCL3/MIP-1α (all from Applied Biosystems), and three endogenous control genes (18S rRNA, cyclophilin A, GAPDH; Applied Biosystems). The primers and probes for CCR1, cyclophilin A and GAPDH are cDNA-specific, whereas the assay for 18S rRNA may detect contaminating genomic DNA. The normalization of CCR1 to any of the three reference genes (housekeeper genes) gave comparable results. The data shown in the text

Table 1. Summary of the biopsies used in the RT-PCR study

Sample name	Gender	Age (years)	Histological diagnosis	Time after Tx (y, m, d)	Crea (mg/dl)	Prot. urine (g/day)	Immuno-suppression
LD1	F	66	LDx		<1.1	<0.2	CS, CyA, MMF
LD2	m	26	LDx w/o prev. damage		0.9	<0.2	CS, CyA, Aza
LD3	m	49	LDx w/o prev. damage		<1.1	<0.2	CS, CyA, MMF
LD4	n.a.	n.a.	LDx w/o prev. damage		<1.1	<0.2	MMF, Tac
CD1	m	50	CDx, minor int. fibrosis		0.9	<0.2	CS, MMF, Tac
CD2	m	54	CDx w/o prev. damage		0.9	<0.2	CS, MMF, Tac
CD3	m	61	CDx w/o prev. damage		1.2	<0.2	CS, MMF
CD4	F	51	CDx, minor int fibrosis		0.7	<0.2	CS, MMF
AR1	F	53	Acute rejection CCTTI	71 d	2.5	0.2	CS, CyA, MMF
AR2	m	33	Acute rejection CCTTI	99 d	3.1	7.0	CS, MMF, Tac
AR3	F	61	Acute rejection CCTTI	90 d	3.0	n.d.	CS, CyA, MMF
AR4	m	49	Acute rejection CCTTI	260 d	5.0	0.3	CS
AR5	m	64	Acute rejection CCTTI	11 d	8.9	n.d.[a]	CS, Tac
AR6	m	63	Acute rejection CCTTI	74 d	8.0	0.4	CS, CyA
AR7	m	66	Acute rejection CCTTII	16 d	n.a.	n.a.	n.a.
AR8	m	32	Acute rejection CCTTII	17 d	6.0	n.d.	CS, CyA, MMF
AR9	F	62	Acute rejection CCTTII	12 d	7.2	n.d.[a]	CS, CyA
AR10	F	44	Acute rejection CCTTII	21 d	9.2	n.d.[a]	CS, MMF
CAN1	m	48	Chronic allograft nephropathy	2 y 1 m 18 d	2.6	0.3	CS, CyA, MMF
CAN2	m	52	Chronic allograft nephropathy	2 y	6.2	1.8	CS, CyA, Aza
CAN3	m	34	Chronic allograft nephropathy	11 y 3 m	2.1	8.0	CS, CyA, MMF
CAN4	m	47	Chronic allograft nephropathy	6 y 11 m	4.4	n.d.	MMF, Tac
CAN5	m	38	Chronic allograft nephropathy	6 y	10.0	7.0	CS, MMF, Tac
CAN6	m	32	Chronic allograft nephropathy	2 y 3 m	1.9	1.5	CS, MMF, Tac
CAN7	m	21	Chronic allograft nephropathy	3 y 11 m	2.1	6.0	CS, MMF
CAN8	F	34	Chronic allograft nephropathy	4 y 1 m	5.6	0.8	CS, MMF

LDx, Living donor; CDx, cadaveric donor; y, years; m, month; d, days; n.d., not determined; n.a., not available; CS, corticosteroids; CyA, cyclosporine A; MMF, mycophenolate mofetil; Tac, tacrolimus.
[a], Anuria.

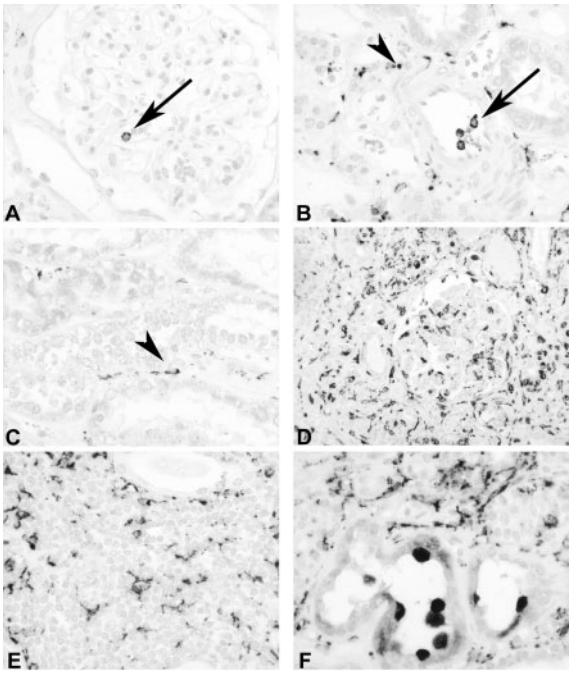

Fig. 2. Immunohistochemistry for CD68 was performed on tumour nephrectomies (**A**–**C**) or allograft nephrectomies (**D**–**F**, orig. ×400). Round, circulating CD68-positive cells were present in glomerular capillaries and interstitial vessels (arrows). Spindle-shaped CD68-positive infiltrating cells were present within the tubulointerstitium (arrowheads). A high number of CD68-positive cells were detectable in allografts with interstitial rejection, both in glomeruli and the tubulointerstitium (D). At sites of nodular inflammatory cell accumulation only scattered CD68-positive cells were present (E). A population of large, round intratubular cells demonstrated prominent CD68 staining (F).

and figures are normalized to 18S rRNA. The biopsy samples used for the mRNA analysis were from a different cohort than the allograft nephrectomy specimens.

Statistical analyses

The statistical analysis was performed using InStat® software (Version 3.05 for Windows, Intuitive Software for Science, San Diego, CA). For the comparison of means, the non-parametric Dunn's multiple comparisons test, and Spearman Rank Correalation was used for the correlations between expression data. $P < 0.05$ was considered to be statistically significant. Statistical analysis was only performed for the mRNA study, not for the immunohistochemical analysis.

Results

CD68 and CCR1 expression in tumour nephrectomies

Ten specimens from tumour nephrectomies were included in this study. The non-involved parts from tumour nephrectomies demonstrated well-preserved renal tissue in eight specimens and prominent interstitial inflammation in two cases. Most of the well-preserved specimens contained only small, focal accumulations of leukocytes, commonly present in nephrectomy specimens. The two nephrectomy specimens with severe interstitial inflammation showed nodular glomerular lesions consistent with diabetic nephropathy (not evaluated as 'normal' controls). These controls were chosen to allow comparison with other nephrectomy tissue; also, interpretation as 'normal' renal tissue is problematic.

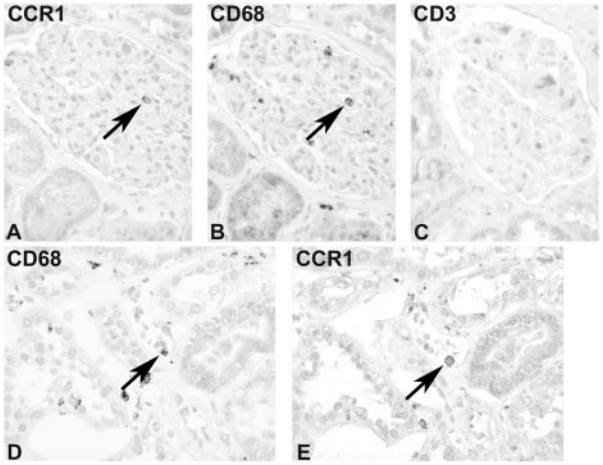

Fig. 3. Immunohistochemistry performed on sections from tumour nephrectomies for CCR1 (**A**, **E**), CD68 (**B**, **D**) and CD3 (C) (A, B, C, orig. ×200; D, E, orig. ×400). A single round CCR1-positive circulating cell can be identified in the glomerular tuft (A), and as CD68-positive on the consecutive section (arrows).

Scattered round circulating CD68-positive cells were present in glomerular capillaries as well as in interstitial vessels in well-preserved renal tissue (Figure 2A and B). In normal glomeruli, the number of CD68-positive cells was low and these cells were predominantly circulating, round CD68-positive monocytes within capillaries. Spindle-shaped CD68-positive cells were present between tubuli and at sites of focal, interstitial leukocyte accumulations (Figure 2C).

CCR1 was found to be mainly expressed by scattered round, circulating cells within glomerular and peritubular capillaries, as well as in larger vessels in well-preserved renal tissue (Figure 3). These cells were CD68-positive on consecutive slides, and therefore corresponded to the CD68-positive cells described earlier (Figure 3A, B, D and E). CCR1-positive cells were rarely found in the tubulointerstitium, even in areas of focal interstitial infiltrates in tumour nephrectomies. Particularly, spindle-shaped CD68-positive cells did not demonstrate CCR1 staining.

In summary, in well-preserved renal tissue CCR1 expression was found on circulating inflammatory cells (consistent with CD68-positive monocytes), but rarely within focal infiltrates, and not on tubular epithelial cells, or intrinsic glomerular cells.

CD68 and CCR1 expression in allograft nephrectomies

Nine specimens from allograft nephrectomies were used in this study. A wide range of lesions was detectable in these specimens. Five specimens showed severe interstitial rejection. One specimen demonstrated vascular rejection in addition to signs of

interstitial rejection. In one specimen signs of chronic allograft nephropathy with glomerulopathy, allograft vasculopathy, interstitial fibrosis and tubular atrophy were present. One specimen demonstrated partial necrosis and interstitial bleeding. One specimen was well preserved with focal interstitial inflammation.

A prominent accumulation of CD68-positive spindle-shaped cells was present within the tubulointerstitium, at times, as multiple layers of cells (Figure 2D). In glomeruli, the number of CD68-positive cells increased as compared with tumour nephrectomies, both as round as well as spindle-shaped cells (Figure 2D). In areas of nodular leukocyte accumulations, scattered CD68-positive cells were detectable with multiple cell processes (Figure 2E). An additional CD68-positive cell population was found within tubular lumina, and at times, in Bowman's spaces. These cells were large, round, without processes and were strongly positive for CD68 (Figure 2F). These cells were found in seven of the nine allograft nephrectomy specimens.

CCR1 expression was also found on circulating cells in arteries, veins, peritubular and glomerular capillaries in allograft nephrectomies, as described for well-preserved renal tissue. CCR1-positive infiltrating cells were present in glomeruli (Figure 4A), consistent with some of the CD68-positive cells (Figure 4B). Most of the prominent spindle-shaped, CD68-positive interstitial cells were negative for CCR1 staining (Figure 4F and G). Scattered CCR1-positive cells were found in the tubulointerstitium. These CCR1-positive cells were mostly negative for CD68 staining either on consecutive sections or by double immunofluorescence (Figure 5A–C). Some of the CCR1-positive interstitial cells were identified as CD20-positive B cells (Figure 5J and K). The number of CCR1/CD20 double-positive cells was small as compared with the overall number of CD20 single-positive cells (Figure 5J).

An additional subpopulation of the CCR1-positive interstitial cells also stained for DC-SIGN (Figure 5G–I). No CCR1 and S100 double-positive cells were detectable. None of the CCR1-positive cells expressed smooth muscle antigen (a marker of myofibroblasts), CD34 (an endothelial marker) or CD3 (a T-cell marker).

A considerable number of round CCR1-positive cells were found at times within Bowman's capsule, and more commonly in tubular lumina (Figure 5D–F). These cells corresponded to the CD68-positive cells described earlier on consecutive tissue sections (Figure 4D–G) or by double immunofluorescence (Figure 5D–F).

In arteriosclerotic lesions of large arteries, CD68-positive macrophages with foam cell appearance were positive for CCR1 (Figure 4H and I). No CCR1 expression was detectable on tubular epithelial cells and intrinsic glomerular cells in renal allografts.

In summary, in renal allografts circulating monocytes, infiltrating CD68-positive cells in glomeruli, within tubular lumina, in arteriosclerotic lesions, as well as some interstitial CD20- or DC-SIGN-positive cells expressed CCR1. No CCR1 expression was found on the majority of spindle-shaped interstitial CD68-positive cells.

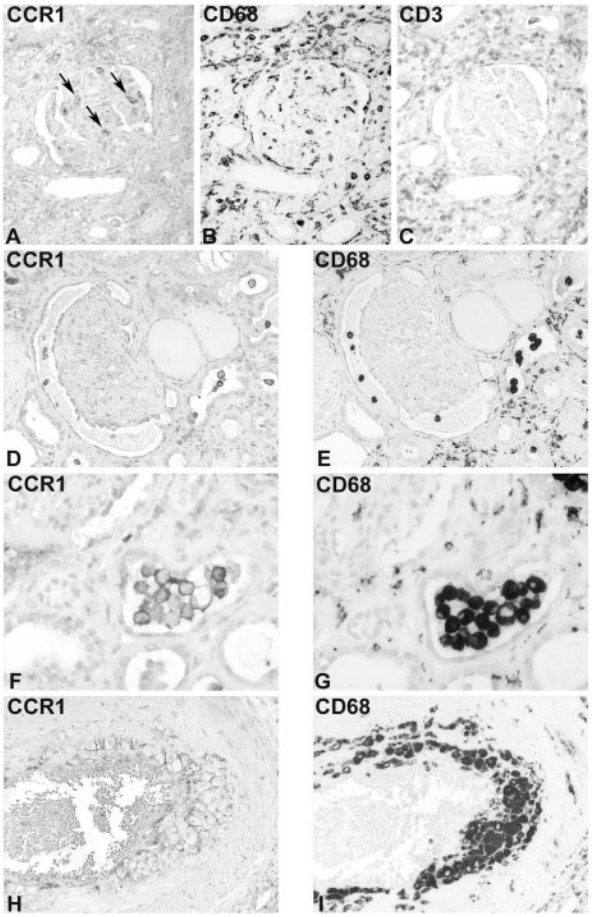

Fig. 4. Immunohistochemistry was performed on allograft nephrectomies for CCR1 (**A**, **D**, **F**, **H**), CD68 (**B**, **E**, **G**, **I**) and CD3 (C, orig X200; A–E, H, I, X400 F, G). CCR1-positive cells are infiltrating the glomerular tuft (arrows), but rarely the tubulointerstitium, whereas CD68-positive cells were present in the interstitium as well as the glomerulus (B). Note the prominent population of CCR1-positive cells within Bowman's space (D), and within tubular lumina (F), which were CD68-positive on consecutive sections (E, G). An arteriosclerotic lesion with CCR1- and CD68-positive foam cells is illustrated in H and I.

Expression of CCR1 mRNA and the ligands CCL5/RANTES and CCL3/MIP-1α in renal allograft biopsies

To quantify the expression of CCR1 and the corresponding ligands in renal biopsies, real-time RT-PCR was used. Biopsies were from a multicentre renal biopsy bank (ERCB), and the available clinical information on these specimens was summarized in Table 1. The mRNA expression was quantified in microdissected tubulointerstitial compartments of renal allograft biopsies with acute cellular rejection (AR), chronic allograft nephropathy (CAN), as well as pre-transplant biopsies (from living and cadaveric donors) as controls. A significant increase in CCR1 mRNA expression was

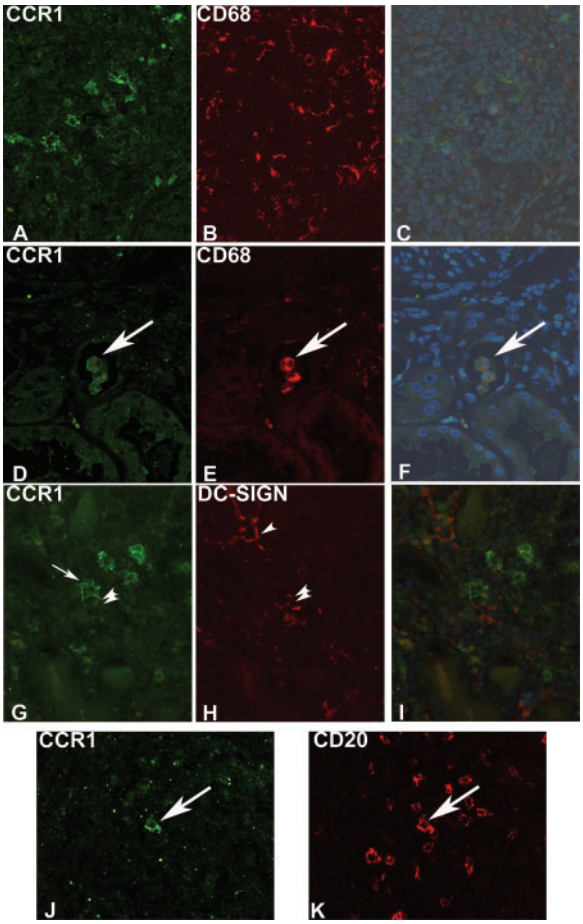

Fig. 5. Double immunofluorescence was performed on allograft nephrectomies for CCR1 and CD68 (**A**–**C** and **D**–**F**), CCR1 and DC-SIGN (**G**–**I**) and CCR1 and CD20 (**J**, **K**, orig X400). C, F and I illustrate an overlay with a nuclear counterstain. Note that there are no CCR1/CD68 double-positive cell in figures A–C. CCR1-positive intratubular cells (D, arrow) which express CD68 (E, arrow) are illustrated in D. CCR1 is expressed by two adjacent cells (G). One (arrow) is single positive for CCR1, and one (double arrowheads) expresses both CCR1 and DC-SIGN. Arrowhead labels DC-SIGN single-positive cells. In J a CCR1/CD20 double positive cell is illustrated (arrow). Note that most of the CD20-positive cells are CCR1 negative (K).

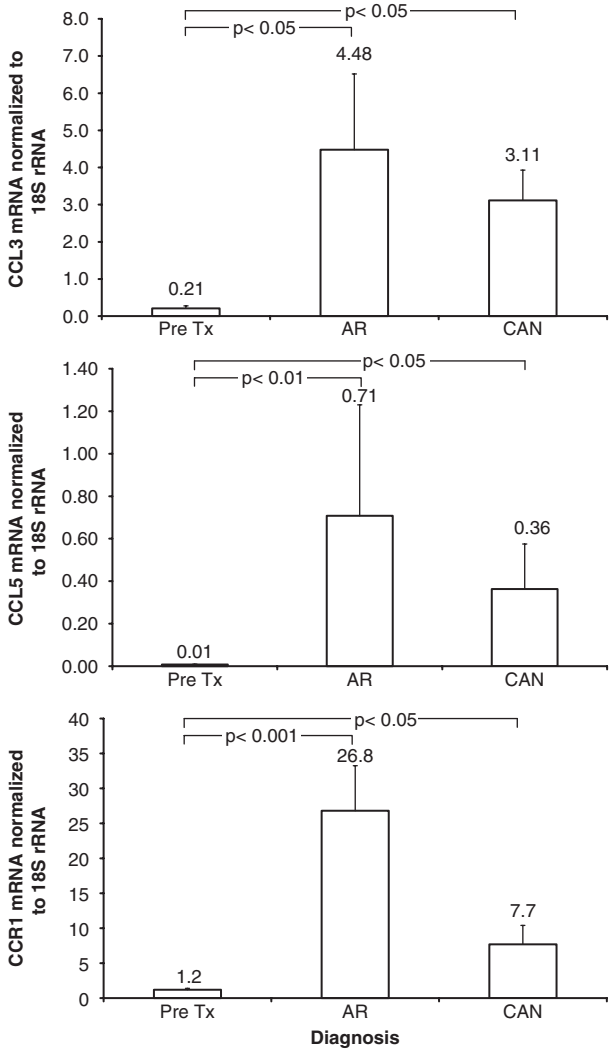

Fig. 6. CCL3/MIP-1α, CCL5/RANTES and CCR1 mRNA expression in renal allograft biopsies. Real-time RT-PCR analysis revealed an induction of the chemokines CCL3 (upper graph), CCL5 (middle graph) and of CCR1 mRNA (lower graph) expression in tubulointerstitial compartments of renal allograft biopsies with acute rejection (AR) and chronic allograft nephropathy (CAN), as compared with pre-transplant controls (Pre Tx).

present in acute allograft rejection, as compared with controls ($P < 0.001$), as well as in biopsies with CAN ($P < 0.05$, Figure 6, lower graph). Correspondingly, significantly higher levels were found for the ligands CCL3/MIP-1α (Figure 6, upper graph) and CCL5/RANTES (Figure 6, middle graph). Additionally, significant correlations between CCR1 mRNA expression and the ligands were found both for CCL3/MIP-1α (Figure 7, upper graph) and CCL5/RANTES (Figure 7, middle graph). Finally, a correlation between CCR1 mRNA and serum creatinine at the time of biopsy was present (Figure 7, lower graph).

Discussion

CCR1 is important for early steps of leukocyte recruitment *in vitro* and might therefore be of particular interest as potential therapeutic target in allograft rejection. In contrast to the detailed animal studies on CCR1 very little is currently known about the expression and distribution of CCR1 in human renal allografts. In this study, we describe an increased expression of CCR1 during allograft injury which was localized to the following distinct cell populations, which will be discussed in detail:

- Circulating CD68-positive monocytes, whereas the majority of interstitial CD68-positive cells were CCR1 negative. Only in specific microenvironments were CD68-positive cells CCR1-positive, i.e. in glomeruli and in arteriosclerotic lesions.
- Scattered CCR1-positive interstitial cells, which were identified as CD20-positive B cells and a

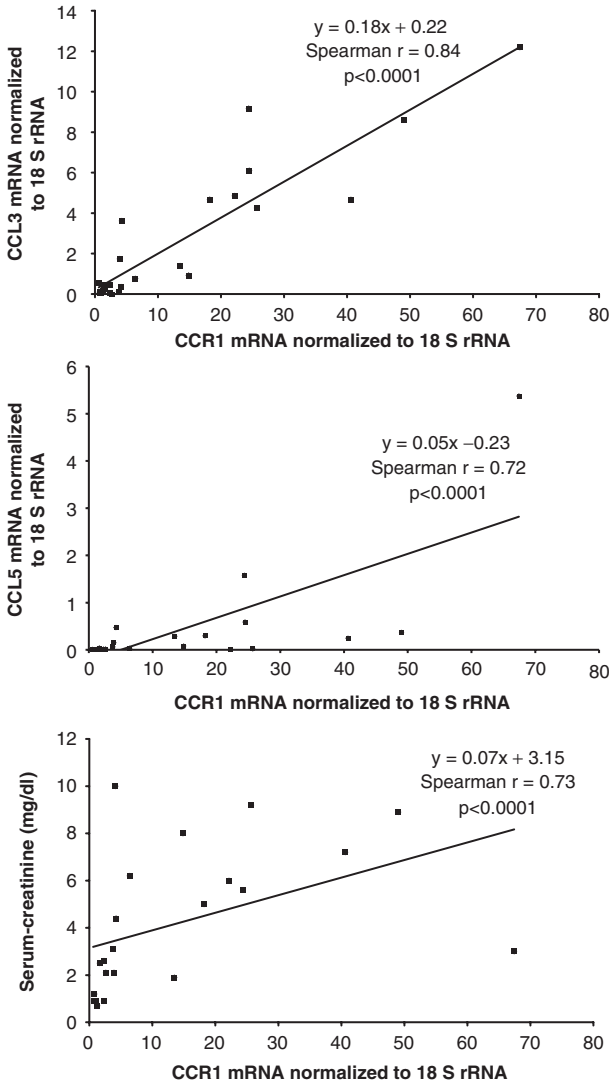

Fig. 7. Correlation between CCR1 mRNA and CCL3/MIP-1α, CCL5/RANTES and serum creatinine. A significant correlation was detectable between CCR1 mRNA and CCL3/MIP-1α (upper graph), CCL5/RANTES (middle graph) and serum creatinine at the time of biopsy (lower graph).

lower number DC-SIGN-positive cells (consistent with immature dendritic cells).

- A population of large round, CD68-positive cells within tubular lumina and Bowman's spaces.

What is known about the role of CCR1 in allograft rejection and renal inflammation? The first evidence of a potential impact of CCR1 blockade came from a mouse heart allograft model [31]. Deficiency of CCR1 prolonged allograft survival and prevented chronic allograft rejection [31]. Met-RANTES, a CCR1 and CCR5 antagonist, also prevented chronic heart allograft rejection in a mouse model [14]. The substance BX-471, a non-peptide CCR1 antagonist, was extensively studied in models of renal inflammation [13] and in renal allograft transplantation [16,32]. BX-471 decreased macrophage recruitment and interstitial fibrosis in unilateral ureter ligation [33,34], in adriamycin nephropathy [35], in a mouse model of lupus nephritis [36] and in a model of Alport's syndrome [37]. In a rabbit renal allograft model, the survival was increased by BX-471 [16]. In a rat renal allograft model, BX-471 reduced inflammatory cell recruitment and improved chronic renal allograft nephropathy [38]. BX-471 has been used in a phase 1 trial for multiple sclerosis [39]. As CCR1 antagonists become available for the treatment of human diseases, it is important to evaluate the CCR1-positive cell types in human diseases and allograft rejection. Using real-time RT-PCR Dalton and colleagues studied chemokine receptor expression in peripheral blood from renal allograft recipients and demonstrated an increased expression of CCR1 in peripheral blood from patients with acute allograft rejection [10]. In human renal allografts, CCR1 mRNA was found to be expressed and CCR1 protein was localized to glomerular macrophages [19,20]. The current study we aimed to give a more detailed description of CCR1-positive cells in renal allografts.

The first population of CCR1-positive cells to be discussed were circulating cells in various renal vascular beds (e.g. glomerular and peritubular capillaries, in arterial and venous lumina). These cells were more commonly found in allografts as compared with tumour nephrectomies and demonstrated a colocalization with CD68-, but not with CD3-positive cells. Therefore, circulating monocytes were commonly CCR1-positive. Using FACS analysis CCR1-positive cells have been studied in peripheral blood of healthy subjects. The numbers vary remarkably. In CD14-positive monocytes, CCR1 expression in controls was described to be between 33 and 90% [40]. In T cells, the percentage was lower with up to 18% [40]. Down-regulation of CCR1 in macrophages has been described under various pathological conditions in peripheral blood, as well as during extravasation, whereas *in vitro* monocytes did not lose CCR1 expression during maturation [41]. Previously, a hierarchy was described in the use of chemokine receptors CCR1 and CCR5 during the recruitment of monocytes/macrophages under flow conditions *in vitro* [11]. CCR1 mediated cell arrest, whereas spreading was CCR5 dependent [11]. Our results are consistent with this model and also with data on CCR1 expression on peripheral blood monocytes and monocytes/macrophages extracted from synovial fluid [12]. Whereas almost all blood monocytes (87%) expressed CCR1, monocytes/macrophages extracted from synovial fluid demonstrated CCR1 expression in only 17% [12].

It is important to note that a loss of CCR1 expression was not true for all renal compartments as CD68-positive cells infiltrating glomeruli and CD68-positive foam cells in arterial walls demonstrated CCR1 expression. Ruster *et al.* [20] localized CCR1 to glomerular macrophages in frozen sections from allograft biopsies. This suggests that monocytes/macrophages differ in their CCR1 expression depending on the renal microcompartments they enter. The glomerular tuft represents a compartment where

inflammatory cells do not penetrate a basement membrane during the extravasation process. This might explain some differences in the composition of the inflammatory cell subsets in this tissue microenvironment. Differences between macrophage populations in different renal compartments have been described during nephrotoxic nephritis in mice [42]. Whereas interstitial macrophages expressed CD68, CD11b and F4/80, only a minority of glomerular macrophages were F4/80 positive [42]. Macrophages can have pro-as well as anti-inflammatory properties, but the chemokine receptor profile of these cell types in renal inflammation are currently not well defined [43].

Another hypothesis could be that a population of interstitial macrophages (primarily CCR1 negative) might increase in number via proliferation. In this case, it would be likely that the interstitium would harbor a mixture of CCR1/CD68-positive cells (recruited) and CCR1-negative macrophages (after local) proliferation, which was not present in our study. Therefore, we favour the hypothesis of CCR1 down-regulation.

Also, previous studies have identified a small population of T cells to express CCR1 and react to CCR1 ligands, we were unable to identify CCR1-positive T cells in renal allografts. As only a minor population of CCR1-positive T cells has been described in peripheral blood, the overall number might be small [44]. The data is consistent with the results by Ruster *et al*. [20] who did not identify a CCR1-positive T-cell population in renal allografts. Another explanation for the absence for CCR1-positive T cells might be that the expression level is to low to detect it by immunohistochemistry. T cells predominantly infiltrate the tubulointerstitium, a down-regulation of CCR1 as suggested in macrophages might therefore also take place in T cells.

We also identified a small population of relatively large interstitial CCR1-positive infiltrating cells, which surprisingly were mainly CD68-negative (as shown by double-labelling and staining of consecutive sections). Some of these CCR1-positive cells were identified as B cells by the marker CD20. CCR1 expression has been previously described in cultured B cells from tonsils, preferentially in non-germinal centre (i.e. naive and memory) B cells, whereas CCR1 was absent on germinal center B cells [45]. In the study here, only a small subpopulation of CD20-positive infiltrating B cells was found to be CCR1-positive. The potential role of CCR1 on some B cells remains speculative at present and may relate to the organization of lymphoid-like aggregates [46]. These B cell containing lymphoid-like aggregates in renal allografts were associated with allograft loss in a pediatric population [47].

Immature dendritic cells are also thought to express CCR1 [48]. Little is currently known about dendritic cells or markers of dendritic cells in human kidney diseases or human renal allografts. DCs generated from monocytes *in vitro* demonstrated CCR1 expression in 10% [49]. Double-labelling studies for DC-SIGN, a marker of immature DCs, identified only a few DC-SIGN-positive cells that also expressed CCR1. No CCR1 expression was found on S100-positive cells, as a marker of more differentiated DCs. Also the absence of CCR1 on differentiated DCs follows the general hypothesis that DCs loose the expression of inflammatory chemokine receptors during differentiation, the number of CCR1-positive immature DCs was low.

An unusual type of cell found to express CCR1 were large, round cells present in tubular lumina and in Bowman's spaces. The source of these cells is not clear. A similar cell population was described in focal-segmental glomerulosclerosis and crescentic glomerulonephritis and was thought to be derived from podocytes. The cells show increased CD68 expression and decreased podocyte markers (such as WT-1) and are shed into the urine [50]. A glomerular source of these CD68-positive cells is suggested by their presence in Bowman's space. Rather than postulating a podocyte origin for them, which would require a total change of phenotype, the possibility has to be considered that they may be CCR1/CD68-positive monocytes/macrophages that have extravasated through the glomerulus.

An increased expression of the CCR1 ligands CCL3/MIP-1α and CCL5/RANTES was found in renal biopsies with acute rejection, but also to a lesser extent in biopsies with chronic allograft rejection. These chemokines have previously been demonstrated during acute rejection both in animal models [51] as well as in human allograft nephrectomies [19]. This is the first study in which a significant correlation between CCR1 and corresponding ligands was demonstrated, particularly for CCL3/MIP-1α. The increased expression of CCR1 and the ligands during CAN is particularly interesting as the treatment with Met-RANTES prevented chronic renal allograft injury in rats [14,15]. These results were found on a relatively small number of patients and should be confirmed in a larger cohort of biopsies.

In summary, CCR1 mRNA expression increases in renal allograft injury, which correlates with the expression of the corresponding chemokine ligands and serum creatinine. We identified distinct populations of inflammatory cells within renal allografts to be CCR1-positive. These include circulating monocytes, CD68-positive cells within glomeruli and in arteriosclerotic lesions, some CD20-positive B cells and some immature dendritic cells. On the other hand the majority of infiltrating CD68-positive spindle-shaped cells did not express CCR1 indicating down-regulation of the receptor in these cells. This study further supports that CCR1 might be a target in human renal allograft injury.

Acknowledgements. S.S. and H.J.A. are supported by Grants of the Else Kröner-Fresenius Stiftung, Germany, the work was supported by grants of the Deutsche Forschungsgemeinschaft (SE 888/4-1, SFB 571 PJN, AN 372/8-1, GRK 1202), the EU Network of

Excellence 'MAIN' (FP6-502935) and the EU integrated project F6 'INNOCHEM'.
Members of the European Renal cDNA Bank (ERCB) at the time of this study: J.P. Rougier, P. Ronco *Paris*; M.P. Rastaldi, G. D'Amico, *Milano*; F. Mampaso, *Madrid*; P. Doran, H.R. Brady, *Dublin*; D. Mönks, C. Wanner, *Würzburg*; A.J. Rees, *Aberdeen*; F. Strutz, G. Müller, *Göttingen*; P. Mertens, J. Floege, *Aachen*; T. Risler, *Tübingen*; L. Gesualdo, F.P. Schena, *Bari*; J. Gerth, U. Ott, G. Wolf, *Jena*; R. Oberbauer, D. Kerjaschki, *Vienna*; B. Banas, B. Krämer, *Regensburg*; W. Samtleben, *Munich*; H. Peters, H.H. Neumayer, *Berlin*; K Ivens, B. Grabensee, *Düsseldorf*; M. Zeier, H.J. Groene, *Heidelberg*; M. Merta, V. Tesar, *Prague*; C.D. Cohen, H. Schmid, M. Kretzler, D. Schlöndorff, *Munich*.

Conflict of interest statement. None declared.

References

1. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; 76: 301–314
2. Segerer S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. *J Am Soc Nephrol* 2000; 11: 152–176
3. Ley K. Arrest chemokines. *Microcirculation* 2003; 10: 289–295
4. Segerer S. The role of chemokines and chemokine receptors in progressive renal diseases. *Am J Kidney Dis* 2003; 41: S15–S18
5. Inston NG, Cockwell P. The evolving role of chemokines and their receptors in acute allograft rejection. *Nephrol Dial Transplant* 2002; 17: 1374–1379
6. Haskell CA, Ribeiro S, Horuk R. Chemokines in transplant rejection. *Curr Opin Investig Drugs* 2002; 3: 399–405
7. Zheng G, Wang Y, Mahajan D *et al*. The role of tubulointerstitial inflammation. *Kidney Int Suppl* 2005; S96–100
8. Colvin BL, Thomson AW. Chemokines, their receptors, and transplant outcome. *Transplantation* 2002; 74: 149–155
9. Murphy PM, Baggiolini M, Charo IF *et al*. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000; 52: 145–176
10. Dalton RS, Webber JN, Pead P, Gibbs PJ, Sadek SA, Howell WM. Immunomonitoring of renal transplant recipients in the early posttransplant period by sequential analysis of chemokine and chemokine receptor gene expression in peripheral blood mononuclear cells. *Transplant Proc* 2005; 37: 747–751
11. Weber C, Weber KS, Klier C *et al*. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells. *Blood* 2001; 97: 1144–1146
12. Katschke KJ,Jr, Rottman JB, Ruth JH *et al*. Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis Rheum* 2001; 44: 1022–1032
13. Anders HJ, Ninichuk V, Schlondorff D. Progression of kidney disease: blocking leukocyte recruitment with chemokine receptor CCR1 antagonists. *Kidney Int* 2006; 69: 29–32
14. Yun JJ, Whiting D, Fischbein MP *et al*. Combined blockade of the chemokine receptors CCR1 and CCR5 attenuates chronic rejection. *Circulation* 2004; 109: 932–937
15. Grone HJ, Weber C, Weber KS *et al*. Met-RANTES reduces vascular and tubular damage during acute renal transplant rejection: blocking monocyte arrest and recruitment. *Faseb J* 1999; 13: 1371–1383
16. Horuk R, Shurey S, Ng HP *et al*. CCR1-specific non-peptide antagonist: efficacy in a rabbit allograft rejection model. *Immunol Lett* 2001; 76: 193–201
17. Racusen LC, Solez K, Colvin RB *et al*. The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55: 713–723
18. Segerer S, Hughes E, Hudkins KL, Mack M, Goodpaster T, Alpers CE. Expression of the fractalkine receptor (CX3CR1) in human kidney diseases. *Kidney Int* 2002; 62: 488–495
19. Segerer S, Cui Y, Eitner F *et al*. Expression of chemokines and chemokine receptors during human renal transplant rejection. *Am J Kidney Dis* 2001; 37: 518–531
20. Ruster M, Sperschneider H, Funfstuck R, Stein G, Grone HJ. Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts. *Clin Nephrol* 2004; 61: 30–39
21. Segerer S, Bohmig GA, Exner M *et al*. When renal allografts turn darc. *Transplantation* 2003; 75: 1030–1034
22. Flugel A, Berkowicz T, Ritter T *et al*. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* 2001; 14: 547–560
23. Shang X, Qiu B, Frait KA *et al*. Chemokine receptor 1 knockout abrogates natural killer cell recruitment and impairs type-1 cytokines in lymphoid tissue during pulmonary granuloma formation. *Am J Pathol* 2000; 157: 2055–2063
24. Sorensen TL, Tani M, Jensen J *et al*. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* 1999; 103: 807–815
25. Segerer S, Banas B, Wornle M *et al*. CXCR3 is involved in tubulointerstitial injury in human glomerulonephritis. *Am J Pathol* 2004; 164: 635–649
26. Segerer S, Henger A, Schmid H *et al*. Expression of the chemokine receptor CXCR1 in human glomerular diseases. *Kidney Int* 2006; 69: 1765–1773
27. Schmid H, Cohen CD, Henger A, Irrgang S, Schlondorff D, Kretzler M. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney Int* 2003; 64: 356–360
28. Schmid H, Cohen CD, Henger A, Schlondorff D, Kretzler M. Gene expression analysis in renal biopsies. *Nephrol Dial Transplant* 2004; 19: 1347–1351
29. Schmid H, Henger A, Cohen CD *et al*. Gene expression profiles of podocyte-associated molecules as diagnostic markers in acquired proteinuric diseases. *J Am Soc Nephrol* 2003; 14: 2958–2966
30. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001; 25: 386–401
31. Gao W, Topham PS, King JA *et al*. Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J Clin Invest* 2000; 105: 35–44
32. Pease JE, Horuk R. CCR1 antagonists in clinical development. *Expert Opin Investig Drugs* 2005; 14: 785–796
33. Anders HJ, Vielhauer V, Frink M *et al*. A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. *J Clin Invest* 2002; 109: 251–259
34. Eis V, Luckow B, Vielhauer V *et al*. Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction. *J Am Soc Nephrol* 2004; 15: 337–347
35. Vielhauer V, Berning E, Eis V *et al*. CCR1 blockade reduces interstitial inflammation and fibrosis in mice with glomerulosclerosis and nephrotic syndrome. *Kidney Int* 2004; 66: 2264–2278
36. Anders HJ, Belemezova E, Eis V *et al*. Late onset of treatment with a chemokine receptor CCR1 antagonist prevents progression of lupus nephritis in MRL-Fas(lpr) mice. *J Am Soc Nephrol* 2004; 15: 1504–1513

37. Ninichuk V, Gross O, Reichel C *et al*. Delayed chemokine receptor 1 blockade prolongs survival in collagen 4A3-deficient mice with Alport disease. *J Am Soc Nephrol* 2005; 16: 977–985
38. Bedke J, Kiss E, Schaefer L *et al*. Beneficial effects of CCR1 blockade on the progression of chronic renal allograft-damage. *Am J Transplant* 2007 (in press)
39. Elices MJ. BX-471 Berlex. *Curr Opin Investig Drugs* 2002; 3: 865–869
40. Talvani A, Rocha MO, Ribeiro AL, Correa-Oliveira R, Teixeira MM. Chemokine receptor expression on the surface of peripheral blood mononuclear cells in Chagas disease. *J Infect Dis* 2004; 189: 214–220
41. Phillips RJ, Lutz M, Premack B. Differential signaling mechanisms regulate expression of CC chemokine receptor-2 during monocyte maturation. *J Inflamm* 2005; 2: 14
42. Masaki T, Chow F, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Heterogeneity of antigen expression explains controversy over glomerular macrophage accumulation in mouse glomerulonephritis. *Nephrol Dial Transplant* 2003; 18: 178–181
43. Kluth DC, Erwig LP, Rees AJ. Multiple facets of macrophages in renal injury. *Kidney Int* 2004; 66: 542–557
44. Bruhl H, Wagner K, Kellner H, Schattenkirchner M, Schlondorff D, Mack M. Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases. *Clin Exp Immunol* 2001; 126: 551–559
45. Corcione A, Tortolina G, Bonecchi R *et al*. Chemotaxis of human tonsil B lymphocytes to CC chemokine receptor (CCR) 1, CCR2 and CCR4 ligands is restricted to non-germinal center cells. *Int Immunol* 2002; 14: 883–892
46. Kerjaschki D, Regele HM, Moosberger I *et al*. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol* 2004; 15: 603–612
47. Sarwal M, Chua MS, Kambham N *et al*. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 2003; 349: 125–138
48. Varani S, Frascaroli G, Homman-Loudiyi M, Feld S, Landini MP, Soderberg-Naucler C. Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5. *J Leukoc Biol* 2005; 77: 219–228
49. Jugde F, Boissier C, Rougier-Larzat N *et al*. Regulation by allergens of chemokine receptor expression on in vitro-generated dendritic cells. *Toxicology* 2005; 212: 227–238
50. Bariety J, Nochy D, Mandet C, Jacquot C, Glotz D, Meyrier A. Podocytes undergo phenotypic changes and express macrophagic-associated markers in idiopathic collapsing glomerulopathy. *Kidney Int* 1998; 53: 918–925
51. Grau V, Gemsa D, Steiniger B, Garn H. Chemokine expression during acute rejection of rat kidneys. *Scand J Immunol* 2000; 51: 435–440

Received for publication: 10.9.06
Accepted in revised form: 4.1.07