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Orginal Article

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Expression of the chemokine receptor CXCR3 in human renal allografts—a prospective study

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Abstract

Background. Mechanisms involved in the recruitment and activation of inflammatory cells during renal allograft injury are still incompletely understood. Since chemokines play pivotal roles in this process, our prospective study was performed to evaluate further the role of the chemokine receptor CXCR3.

Methods. A total of 138 biopsies were included from patients without rejection and unaltered morphology (according to Banff 97 classification grade 1, $n=49$), with acute interstitial rejection (Banff grade 4 type I, $n=8$), with acute vascular rejection (Banff grade 4 type II, $n=23$), with chronic allograft nephropathy (Banff grade 5, $n=16$), without rejection but with various other lesions (Banff grade 6, $n=36$) and from pre-transplant kidneys ($n=6$). The expression of CXCR3-, CD4- and CD8-positive cells was localized by immunohistochemistry and quantified by image analysis.

Results. CXCR3 was expressed by infiltrating inflammatory cells, but not by intrinsic renal structures. CXCR3-positive cells were found to be involved in tubulitis and vascular rejection. The area of CXCR3-positive staining was significantly larger in biopsies with acute interstitial rejection ($P<0.001$) and acute vascular rejection ($P<0.001$) as compared with normal renal graft biopsies. There was a strong morphological and numerical correlation between CXCR3 and both CD4- and CD8-positive T cells, respectively.

Conclusions. A significant part of both CD4- and CD8-positive T cells express the chemokine receptor CXCR3. During renal allograft rejection, the number of these cells increases significantly at the site of injury and might be targeted by CXCR3 blocking agents.

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Introduction

Acute renal allograft rejection is characterized by an inflammatory cell recruitment to different renal compartments [1]. T lymphocytes are of major importance for acute rejection and for allograft survival [1,2]. Subpopulations of inflammatory cells are guided towards specific renal microenvironments in a complex cascade of interactions between adhesion molecules, chemokines and chemokine receptors [3–5]. Chemokines are members of a large family of chemotactic cytokines, which play pivotal roles during leukocyte recirculation, lymphatic microenvironment formation, and acute and chronic inflammatory processes [6–11]. CXCR3 is a receptor for monokine induced by interferon (IFN)-γ (Mig/CXCL9), interferon-inducible protein 10 (IP-10/CXCL10) and IFN-inducible T-cell α-chemoattractant (I-TAC/CXCL11) [6]. Expression of CXCR3 has been demonstrated on circulating T cells, with a strong induction of CXCR3 after T cell activation [12]. A major importance of CXCR3 has been described in transplantation models, e.g. heart and bowel transplantation in the mouse, and a model of graft *vs* host disease [4,13,14]. In renal biopsies from patients with glomerular diseases, the number of CXCR3-positive interstitial inflammatory cells (mainly T cells) correlates well with renal function [15]. In serial biopsies from human heart allografts, it was demonstrated that expression of CXCR3 and its ligands correlates with severity of rejection, and decreases after successful treatment [16]. The CXCR3 ligands IP-10/CXCL10 and MIG/CXCL9 are differentially involved in heart transplant pathology as MIG/CXCL9 was associated with chronic allograft

vasculopathy and IP-10/CXCL10 increased during acute rejection [17].

In renal allografts, the ligand IP-10/CXCL10 has been shown to be increased during rejection [18,19]. The CXCR3 ligands are excreted in the urine of renal allograft recipients during allograft rejection and might, therefore, become useful for allograft monitoring [20–22]. Furthermore, high pre-transplant serum IP-10/CXCL10 levels were reported to be associated with severe acute renal rejection, chronic allograft rejection and lower 5-year kidney graft survival rates [23]. Increased CXCR3-positive cells have been demonstrated in allograft biopsies with acute allograft rejection and chronic allograft glomerulopathy [24–26]. To characterize further the role of CXCR3 in human renal allograft rejection, we performed the current prospective study.

Subjects and methods

Patients and renal biopsies

The study period was from July 2003 to June 2004. Protocol biopsies were performed 2 weeks and 3 months after transplantation. Additional biopsies were taken during allograft dysfunction. During this study period, 148 renal allograft biopsies were performed in our transplantation centre. Ten cases had to be excluded due to limitations of renal tissue or due to invalid staining. A total of 138 formalin-fixed, paraffin-embedded renal specimens were included in the analysis. The biopsies were graded according to the Banff 97 working classification [1] by a single pathologist. C4d staining for antibody-mediated rejection identification was not performed routinely, but only in 19 cases where humoral rejection was considered. Clinical data related to the kidney biopsy samples were derived from all renal transplant recipients of our centre. Human tissue was used following the guidelines of the Ethics Committee of the Medical Faculty of the University of Regensburg, Germany.

Immunohistochemistry

Immunohistochemistry for CXCR3 was performed as previously described [15]. Sections (4 μm) were deparaffinized and rehydrated. Endogenous peroxidases were blocked by hydrogen peroxide, and antigen retrieval was performed by microwave treatment in Antigen Unmasking Solution (Vector, Burlingame, CA). Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector). The monoclonal anti-human CXCR3 antibody (clone 1C6, BD Biosciences Pharmingen, San Diego, CA) was used at 10 μg/ml in 10% non-fat dry milk. After subsequent washing steps, the tissue was incubated with the biotinylated secondary antibody (Vector). For signal amplification, the ABC-Elite reagent (Vector) was used. 3,3′-Diaminobenzidine (DAB) with nickel enhancement, resulting in a black coloured product, served as chromogen. Slides were counterstained with methyl green, dehydrated and coverslipped.

Additionally, slides were stained for CD4- and CD8-positive T cells in an automated slide stainer. Antigen retrieval was performed in citrate buffer by microwave treatment. Incubation of the primary antibodies was for 24 min [anti-CD4 (1F6), Ventana, Strasbourg, France] and 32 min [anti-CD8 (1A5), Ventana], respectively. This was followed by incubation with a biotinylated secondary antibody (Ventana) for 8 min. DAB (Ventana) with copper enhancement was used as the detection system, resulting in a brown coloured product. Slides were counterstained with haematoxylin, dehydrated and coverslipped. Due to limitations in biopsy material from the prospectively collected biopsies, additional serial sections from allograft biopsies with defined disease entities, and sections from human tonsils and human tumour nephrectomies were used to define the CXCR3-positive cell population further, as well as to serve as additional positive and negative controls (isotype controls). Staining for CXCR3-, CD3-, CD4- and CD8-positive T cells (rabbit anti-human, A0452; Dako, Carpenteria, CA) was performed on serial sections on these tissues [15]. Furthermore, we used double fluorescence in selected biopsies to define the CXCR3-positive cell population on formalin-fixed, paraffin-embedded materials. The protocol was similar to that described above. To combine CXCR3 and CD8 staining, incubation with a biotinylated anti-mouse IgG1 (Vector) was followed by exposure to streptavidin–fluorescein isothiocyanate (FITC) (Dako, Glostrup Denmark). After microwave treatment, CD8 was detected by an anti-mouse antibody conjugated to Texas red (Vector).

For the combination of CXCR3 with CD4, CXCR3 was detected by anti-mouse Cy3-conjugated antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and CD4 with a biotinylated anti-mouse antibody (Vector) and streptavidin–FITC (Dako). As controls, each staining series consisted of four consecutive sections, replacing each primary antibody with the isotype IgG control. This results in single staining (when one antibody is replaced) or negative staining (after replacement of both antibodies). This excludes cross-reaction of the secondary antibodies.

Digital imaging and statistics

Morphometric analysis was performed in 10 consecutive high power fields (original magnification ×400, covering an area of 296 μm × 222 μm) by the use of Meta View software (Leica, Bensheim, Germany), with a cut-off chosen to eliminate background staining. The area of positive signal was measured for CXCR3, CD4 and CD8. Biopsies without any positive cells were excluded from the analysis, as invalid staining cannot be excluded. Mean values were calculated and compared between different disease entities. For the comparison of means, the non-parametric Mann–Whitney *U*-test was used. $P < 0.05$ was considered to be statistically significant.

Results

Immunohistochemistry, using the monoclonal antibody 1C6 has been used previously to localize CXCR3-positive cells in formalin-fixed, paraffin-embedded renal biopsies [15]. This antibody results in a very reliable staining pattern with very low background (Figure 1A). Negative controls in the form of isotype-matched IgG did not demonstrate positive staining (Figure 1E). A positive colour product was found

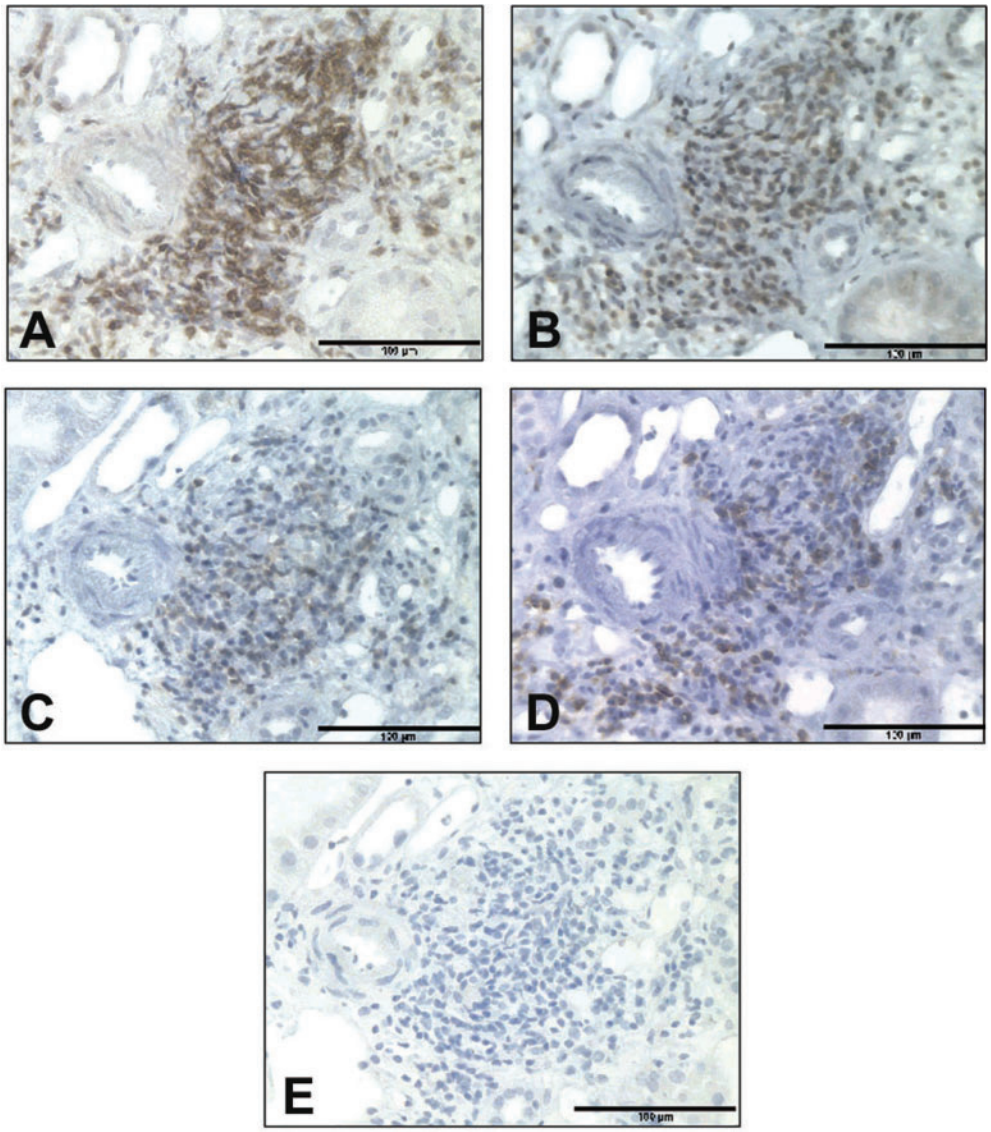

Fig. 1. Immunohistochemistry using antibodies against CXCR3 (**A**), CD3 (**B**), CD4 (**C**), CD8 (**D**) and an isotype IgG (**E**) performed on consecutive sections of a renal allograft biopsy with acute interstitial rejection (original magnification ×400). Note the similar pattern of CXCR3 (A) and CD3 (B), which reflects both CD4- (C) and CD8- (D) positive T cells. No colour product can be detected in the isotype control (E).

exclusively on infiltrating cells, but not intrinsic renal structures (Figure 1A). In particular, intrinsic cells of arteries and glomeruli were CXCR3 negative (Figures 1A and 2A).

Generally, CD4-positive T cells outnumbered CD8-positive T cells (Figures 1C and D). The mean area of positive coloured product for CXCR3- CD4- and CD8-positive cells is shown in Figure 3. The number and distribution of CXCR3-positive cells co-localized with a significant part of both CD4- and CD8-positive T cells, and with the majority of CD3-positive T cells (Figure 1A–D).

Normal renal graft biopsies

Six pre-transplant biopsies from six donors and 49 biopsies from 37 patients demonstrated no signs of rejection and well-preserved tissue (classified as Banff grade 1, Table 1). Only scattered CXCR3-positive cells in peritubular capillaries, at sites of focal interstitial infiltrates, and occasionally in glomerular capillaries, were detected in these biopsies. CXCR3-positive cells were rarely found within glomerular tufts (Figure 2). The area involved in positive staining covered a mean of 0.77% (CD4, 0.65%; CD8, 0.54%; Figures 2 and 3).

Acute interstitial rejection

Eight biopsies from seven patients were classified as Banff grade 4 type I, demonstrating signs of acute interstitial allograft rejection (Table 1). This was the group with the most prominent CXCR3-positive

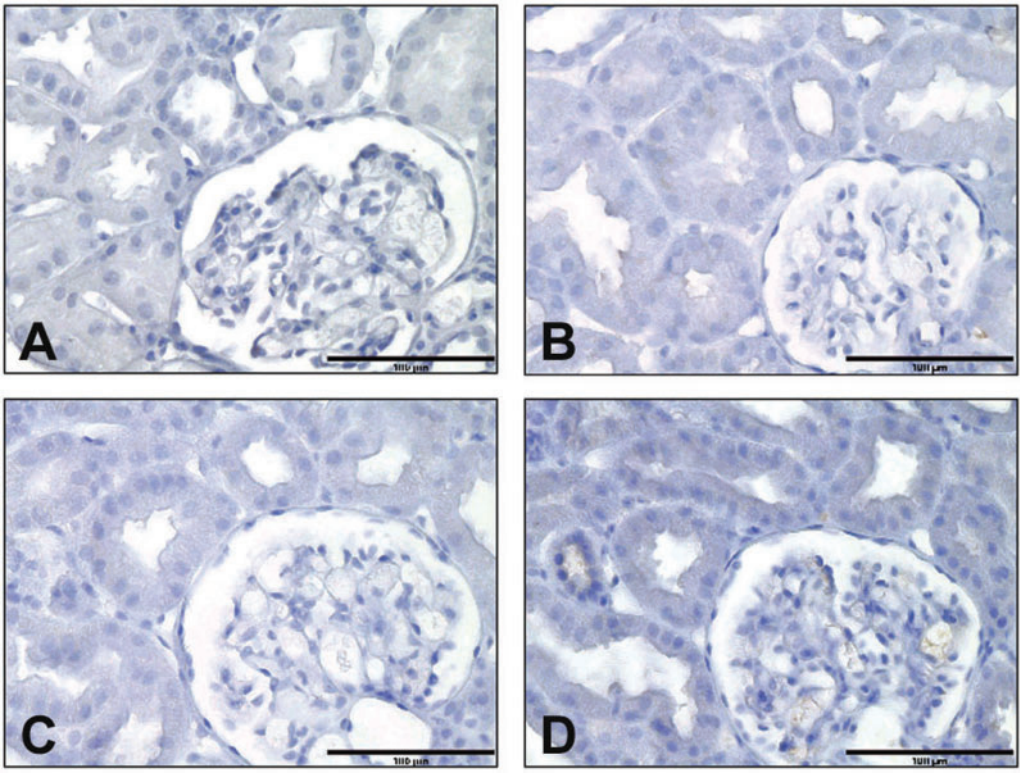

Fig. 2. Immunohistochemistry using antibodies against CXCR3 (**A**), CD3 (**B**), CD4 (**C**) and CD8 (**D**) performed on consecutive sections of a normal renal graft biopsy (original magnification ×400). Note the low number of positive cells.

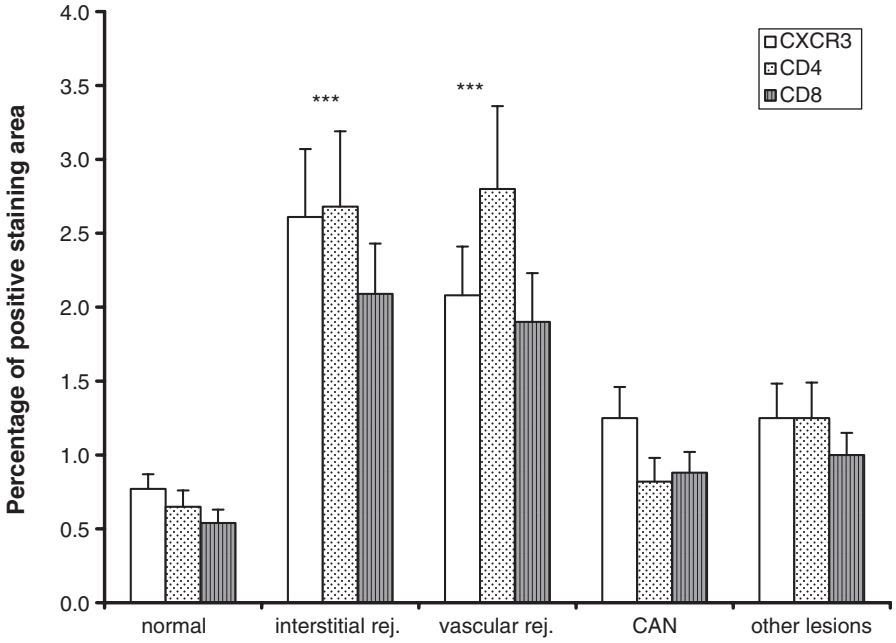

Fig. 3. Mean area of positive colour product for CXCR3-, CD4- and CD8-positive cells. Renal transplant biopsies with normal renal graft pathology (normal), without rejection but with various other lesions (other lesions), with acute interstitial rejection (interstitial rej.), with acute vascular rejection (vascular rej.) and with chronic allograft nephropathy (CAN); $***P < 0.001$ *vs* no rejection.

cell infiltrates (Figure 1). CXCR3-positive cells accumulated in the interstitium between tubuli (Figure 1A) and were found to be involved in tubulitis. The area of positive staining for CXCR3 covered a mean of 2.45% (CD4, 2.08%; CD8, 1.09%) and was significantly larger compared with normal renal allograft biopsies ($P < 0.001$, Figure 3).

Table 1. Demographic data of the study population [mean ± SD, (median)]

	Normal renal graft biopsies ($n = 55$)	Acute interstitial rejection ($n = 8$)	Acute vascular rejection ($n = 23$)	Chronic allograft nephropathy ($n = 16$)	Without rejection, but with other lesions ($n = 36$)
Age	52 ± 15 (55)	57 ± 14 (63)	56 ± 8 (54)	58 ± 10 (58)	65 ± 12 (60)
Sex (male/female)	40/15	6/2	19/2	10/6	17/19
Time of renal biopsy after transplantation (days)	66 ± 78 (63)	59 ± 38 (90)	38 ± 30 (26)	1482 ± 1019 (1460)	212 ± 500 (18)

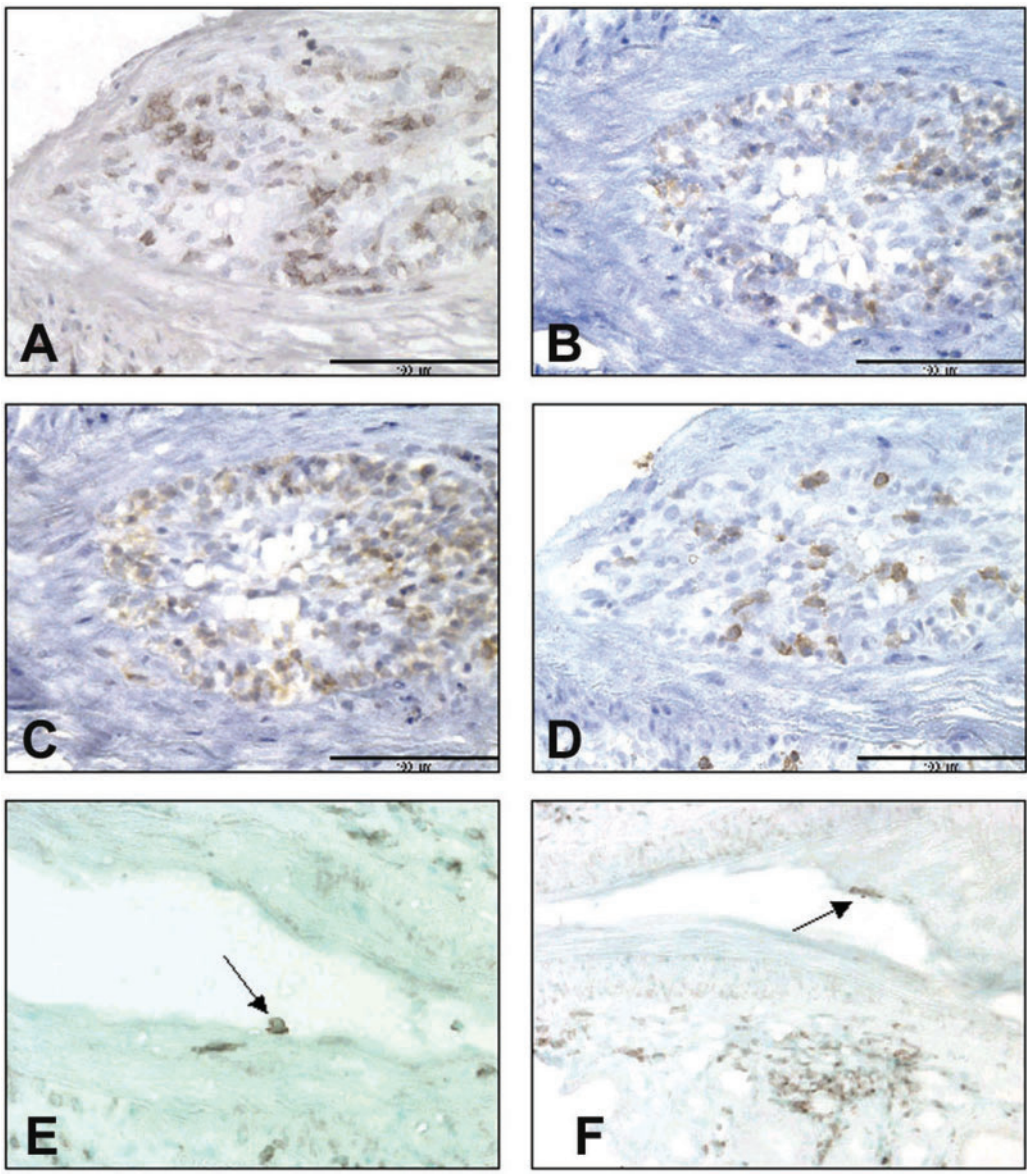

Fig. 4. Immunohistochemistry using antibodies against CXCR3 (**A, E** and **F**), CD3 (**B**), CD4 (**C**) and CD8 (**D**) performed on a renal allograft biopsy with acute vascular rejection (original magnification ×400). Note the CXCR3-positive cell attached to the arterial endothelium (E, arrow), and in the subendothelial area (F, arrow).

Acute vascular rejection

Acute vascular rejection was present in 23 biopsies from 12 patients (17 biopsies classified as Banff grade 4 type IIA, five as Banff grade 4 type IIB, one as Banff grade 4 type III, Table 1). An accumulation of inflammatory cells in the subendothelial area of arteries was present in these cases, containing CXCR3-positive infiltrating cells (Figure 4A and F). CXCR3-positive cells were found to be attached to the arterial endothelium (Figure 4E). The area of CXCR3-positive staining was not significantly different from that in biopsies

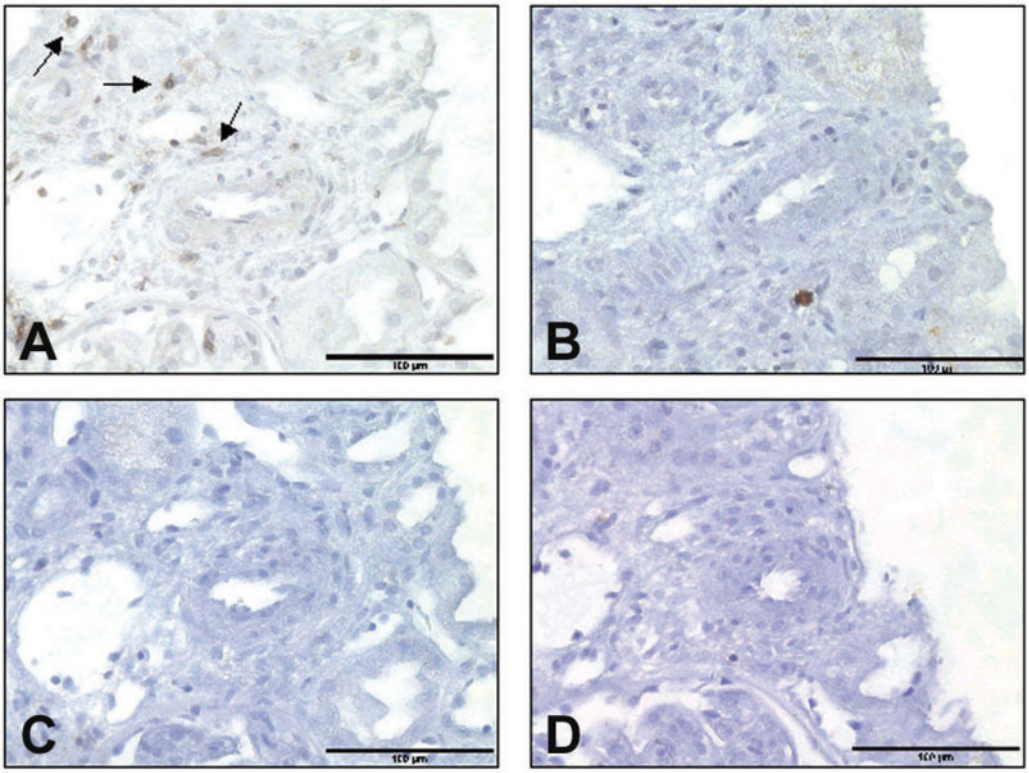

Fig. 5. Immunohistochemistry using antibodies against CXCR3 (**A**), CD3 (**B**), CD4 (**C**) and CD8 (**D**) performed on a renal allograft biopsy with chronic allograft nephropathy (original magnification ×400).

with interstitial allograft rejection, but was significantly larger than in normal renal allograft biopsies ($P < 0.001$). CXCR3-positive staining covered a mean of 2.23% (CD4, 3.00%; CD8, 2.00%; Figures 3 and 4).

Chronic allograft nephropathy

Sixteen biopsies from 14 patients, with a mean time period after transplantation of 1481 days, demonstrated signs of chronic allograft nephropathy (CAN, e.g. vasculopathy, interstitial fibrosis and tubular atrophy; seven classified as Banff grade 5 type I, and nine as grade 5 type II, Table 1). The diffuse, at times patchy interstitial infiltrates contained CXCR3-positive cells (Figure 3). However, overall, the area of positive staining for CXCR3 was comparativey small, with 1.25% CXCR3-positive staining (CD4, 0.82%; CD8, 0.88%; Figures 3 and 5).

Biopsies without rejection, but with various other lesions

This group included 36 biopsies from 29 patients (Table 1). These biopsies did not fulfil the criteria for allograft rejection and were classified as Banff grade 6, but demonstrated either significant tubular injury (clinically commonly associated with acute renal failure), an influx of neutrophils (at times consistent with pyelonephritis) and severe atherosclerosis (commonly containing some sclerosed glomeruli with focal inflammatory infiltrates). Depending on the composition of the inflammatory cell infiltrates, the biopsies varied in numbers of CXCR3-positive cells (Figure 3). CXCR3-positive infiltrating cells were found in areas with chronic interstitial lesions even in the absence of acute rejection. The mean percentage of positive staining area for CXCR3 in these disease entities was 1.25% (CD4, 1.25%; CD8, 1.0%; Figure 3).

Comparison of CD4, CD8 and CXCR3

For each biopsy, the area of positive colour product was quantified by morphometric analysis (Figure 3). As described in the morphological results, the area covered by CD4-positive cells was larger than the area of positive coloured product for CD8, but the difference did not reach statistical significance. In biopsies without signs of rejection and in biopsies with acute interstitial rejection, the area covered by CXCR3 was larger than the area of either CD4- or CD8-positive cells. This finding supports the morphological result that both cell types can express CXCR3. The area of CXCR3-, CD4- and CD8-positive T cells increased during both acute interstitial and acute vascular allograft rejection. In biopsies with vascular rejection, the area of CD4-positive staining was larger than the CXCR3-positive area, indicating a CXCR3-negative CD4-positive T cell population in this disease entity. We performed double staining for CXCR3, CD4 and CD8 (Figure 6). The pattern of CD4 and CXCR3 was very similar (Figure 6A and B), whereas CD8-positive cells were

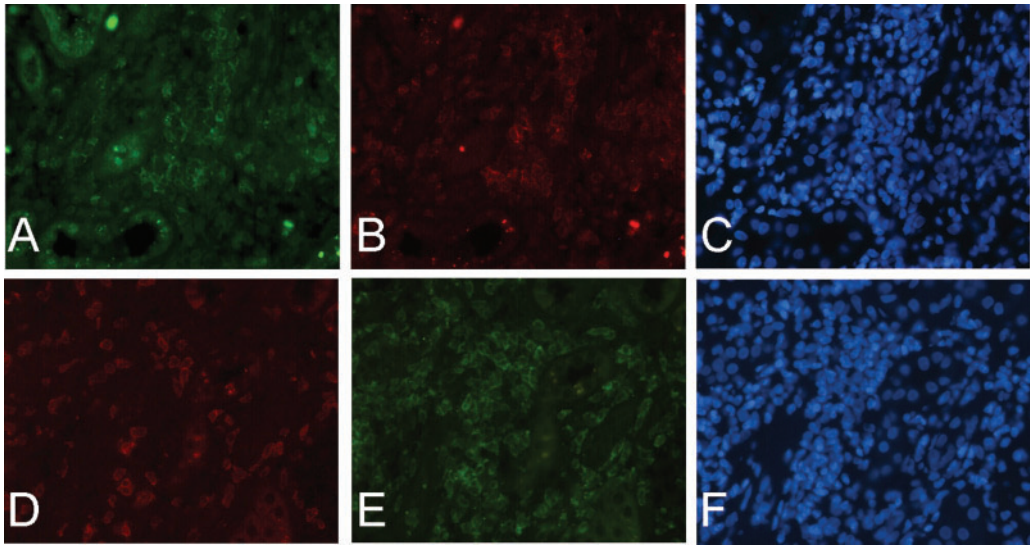

Fig. 6. Double immunofluorescence for CD4/CXCR3 (**A** and **B**) and CD8/CXCR3 (**D** and **E**, nuclei are counterstained with DAPI in **C** and **F**). A renal biopsy was stained for CD4 (**A** in green) and CXCR3 (in red B, ×400). A consecutive staining pattern is demonstrated in (A) and (B), illustrating that most CD4-positive cells express CXCR3. In contrast, the number of CD8-positive cells (D, in red) is outnumbered by those expressing CXCR3 (E, in green), indicating that a smaller population of CXCR3-positive cells are CD8 positive.

outnumbered by CXCR3-positive cells. These results demonstrate that the majority of CXCR3-positive cells infiltrating the kidney are CD4-positive cells, whereas a smaller population of CXCR3-positive cells are CD8-positive T cells.

Correlation of positive C4 deposition and CXCR3 staining

C4d staining for antibody-mediated rejection identification was performed in 19 cases, where humoral rejection was considered. In the group of acute interstitial rejection, one of five biopsies was positive, in the group of acute vascular rejection, one of six biopsies was positive, and in the group of CAN, two out of eight biopsies were positive for C4d staining. The mean percentage of positive CXCR3 staining was 2.2% with negative C4d staining and 1.2% with positive C4d staining ($P = 0.1$).

Clinical outcome

The area of positive coloured product for CXCR3 was not associated with serum creatinine concentration (on the day of biopsy, and 14 days, 3, 6, 9 and 12 months after transplantation). All patients with acute interstitial and acute vascular rejection were treated with high dose steroids (250 mg prednisolone) for 3 days. In one patient with acute interstitial rejection and in 12 patients with acute vascular rejection, a second biopsy was available for analysis. In the patient with acute interstitial rejection, CXCR3-positive staining decreased after the prednisolone treatment (0.72% before and 0.04% after treatment). In the group with acute vascular rejection, the area of CXCR3-positive cells decreased significantly (1.61% before and 0.93% after treatment, $P = 0.04$). Six of the 13 patients were steroid responsive and seven of the 13 patients were steroid resistant. When correlating the CXCR3 levels with steroid responsiveness, there was no significant difference between groups.

Discussion

Acute renal allograft rejection, BK virus nephropathy and CAN are major hurdles for successful long-term renal transplant function. In all these entities, the recruitment of certain subpopulations of inflammatory cells into the vascularized allograft is of major importance.

In contrast to animal experiments, only a few studies to date have been performed to examine the role of CXCR3 and its ligands in human renal allografts. The current study is the largest prospective evaluation to date characterizing CXCR3-, CD4- and CD8-positive cells in renal allograft biopsies. Included were 138 kidney transplant biopsies to localize CXCR3-positive cells by immunohistochemistry. In concordance with other studies, a positive coloured product was found exclusively on infiltrating cells but not on intrinsic renal structures, particularly not on intrinsic cells of arteries and glomeruli [15,21,25].

Biopsies with acute interstitial allograft rejection demonstrated the most prominent accumulation of CXCR3-positive infiltrating cells. This is consistent with published data demonstrating dense staining for IP-10/CXCL10 of renal tubules and adjacent interstitial cells, and CXCR3-positive mononuclear cells in areas of tubulitis [21]. CXCR3-positive cells diffusely infiltrate the interstitium between tubuli as well as the tubular epithelium [25,26]. Accordingly, anti-rejection

treatment with high doses of steroids rapidly decreased the area of CXCR3 staining in biopsies with acute interstitial allograft rejection.

A role for CXCR3-positive cells during vascular rejection has not been described previously. In renal biopsies with acute vascular rejection, an accumulation of CXCR3-positive inflammatory cells was present in the subendothelial area of arteries. The area of CXCR3 staining was not different from that in biopsies with acute interstitial rejection. Anti-rejection treatment with pulse steroids also significantly decreased the area of CXCR3-positive staining in biopsies with acute vascular rejection.

In contrast to previous studies reporting negative staining for CXCR3 in biopsies without signs of rejection, we demonstrate that even in the absence of rejection, CXCR3-positive infiltrates are commonly present in renal allograft biopsies [21]. The biopsies of our patients without rejection and well-preserved tissue contained a low number of scattered CXCR3-positive cells in interstitial peritubular capillaries, at focal sites of interstitial infiltrates and occasionally in glomerular capillaries [25]. The area of CXCR3 staining is larger during rejection, but there is a large overlap between the groups. Therefore, these results question a significant role for CXCR3 staining as a diagnostic marker for rejection, but our study design is not sufficient to resolve this issue.

In the 16 biopsies of patients with signs of CAN (e.g. vasculopathy, interstitial fibrosis and tubular atrophy), the diffuse, at times patchy interstitial infiltrates, contained a significant number of CXCR3-positive cells. In contrast to the study by Akalin *et al.*, we did not detect a prominent number of intraglomerular CXCR3-positive cells in this patient group [24]. In this study, CXCR3-positive cells were found in glomeruli in all biopsies with chronic transplant glomerulopathy, whereas only minor staining of interstitial leukocytes in the remainder of each biopsy was noted [24].

Panzer *et al.* studied serial sections for CXCR3, CD3, CD4, CD8, CD20 and CD68 [25]. The vast majority of CXCR3-positive cells were identified as CD4-positive and the minority as CD8-positive T lymphocytes, consistent with our data. We found in our biopsies that the area stained by CD4-positive cells increased in both types of rejection, and in vascular rejection became larger than the area covered by CXCR3-positive cells. This finding indicates that a CXCR3-negative, CD4-positive cell type increases in number during acute vascular rejection. By double staining for CXCR3, CD4 and CD8, we demonstrated that the pattern of CD4 and CXCR3 was very similar whereas CD8-positive cells were outnumbered by CXCR3-positive cells. These results suggest that the majority of CXCR3-positive cells infiltrating the kidney are CD4-positive cells, whereas a smaller population of CD8-positive cells express CXCR3.

In our study, only 19 biopsies were stained for C4d: four biopsies were C4d positive and 15 were C4d negative. Correlating the positive- and negative-C4d staining with the percentage of CXCR3-positive staining yielded no association. However, the number of C4d-stained biopsies was very small.

It has been shown in previous studies that during episodes of cardiac [27–29], lung [30], skin [31] and liver [32] allograft rejection, CXCR3 and its ligands are strongly associated with rejection. These data are consistent with a role for CXCR3 and its ligands in the recruitment of lymphocytes to sites of rejection in various organs. In a major histocompatibility complex mismatched heart allograft model, allograft recipients lacking CXCR3 demonstrated a profound delay in development of allograft rejection (55–60 days *vs* 7 days in wild-type mice) [33]. In line with these findings, heart allografts from IP-10/CXCL10$^{-/-}$ donors demonstrated significantly prolonged survival as compared with wild-type donors [28]. These results still await confirmation by other groups. The efficacy of targeting donor-derived IP-10/CXCL10 production was confirmed in a heterotopic small bowel allograft model [13]. Additionally, therapy with an anti-CXCR3 monoclonal antibody has been shown to prevent recruitment of activated T cells to the graft and reversed the development of acute allograft rejection in rodent models [33]. In addition to the therapeutic approaches of targeting this pathway using knockout mice or blocking monoclonal antibodies, antisense therapy, directed at CXCR3 [33] or blockade of cytokine and chemokine binding to local glycosaminoglycans [34], has proven modestly effective.

In addition to the above-mentioned therapeutic considerations, urinary IP-10/CXCL10, Mig/CXCL9 and I-TAC/CXCL11, and CXCR3 mRNA have been proposed as biomarkers of acute renal allograft rejection [22]. Using a highly sensitive and specific urinary assay, Hu *et al.* found elevations of urinary IP-10/CXCL10, Mig/CXCL9 and I-TAC/CXCL11 in renal allograft recipients with acute rejection, acute tubular injury and BK virus nephritis when compared with recipients with CAN, stable graft function and healthy individuals [20]. Urinary levels of IP-10/CXCL10 and Mig/CXCL9 declined after the initiation of anti-rejection therapy, consistent with our data [20]. Tatapudi *et al.* reported similar findings regarding mRNA elevations for IP-10/CXCL10 and CXCR3 in urine of patients with acute renal allograft rejection [21]. In a recent study, high *vs* low pre-transplant IP-10/CXCL10 serum levels have been correlated with the risk of acute rejection, chronic allograft rejection and allograft failure in 316 renal graft recipients, and therefore was suggested to be a clinically useful parameter [23].

In conclusion, in this prospective study, the number of CXCR3-positive cells (which resemble both CD4- and to a lesser degree CD8-positive T cells) increases significantly at the site of allograft rejection. The area of CXCR3-positive staining decreased after steroid pulse therapy. A smaller number of CXCR3-positive cells can be detected in the tubulointerstitium during other allograft pathologies. Therefore, CXCR3 blockade may be considered as an attractive therapeutic approach in human renal transplantation.

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U.H. and S.S. contributed equally to this work.

Conflict of interest statement. None declared.

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