The chemokine receptor CXCR7 is expressed on lymphatic endothelial cells during renal allograft rejection

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CXCR7 is an atypical receptor for the chemokines CXCL11 and CXCL12, which were found to be involved in animal models of allograft injury. We studied the expression of CXCR7 and its ligands in human kidneys by first quantifying the mRNA in 53 renal allograft biopsies. Receptor and ligand mRNAs were expressed in renal allografts, with a significant induction of CXCL11 and CXCL12 in biopsies showing borderline lesions and acute rejection. Immunohistochemical analysis for CXCR7 was performed in a series of 64 indication and 24 protocol biopsies. The indication biopsies included 46 acute rejections, 6 with interstitial fibrosis and tubular atrophy, and 12 pretransplant biopsies as controls. In control biopsies, CXCR7 protein was found on smooth muscle and on endothelial cells of a small number of peritubular vessels. The number of CXCR7-positive vessels was increased in acute rejection and, using double immunofluorescence labeling, a subset of these CXCR7-positive endothelial cells were identified as lymphatic vessels. Both CXCR7-positive blood and lymphatic vessels increased during allograft rejection. We found that CXCR7 is present in both blood and lymphatic endothelial cells in human renal allografts. Whether its presence modulates the formation of chemokine gradients and the recruitment of inflammatory cells will require further experimental studies.

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Recruitment of inflammatory cells has a major function for allograft survival in almost all forms of renal allograft injury ranging from ischemia reperfusion, acute allograft rejection to chronic injury (interstitial fibrosis and tubular atrophy).[1,2] Chemokines are members of a large family of chemotactic cytokines, which are involved in organogenesis, organization of the immune system, the formation of lymphoid tissue, leukocyte recirculation, and the recruitment of inflammatory cells to vascularized allografts.[2–5]

CXCR7 is a recently de-orphanized receptor (also known as RDC1). It was first cloned according to its homology with the conserved domain of G-protein-coupled receptors.[6] Binding of radiolabeled CXCL12 to embryonic liver cells from CXCR4-deficient mice led to the description of a second receptor for CXCL12.[7–9] CXCR7 also binds CXCL11 with high affinity.[7–9]

CXCR7 is an 'atypical' chemokine receptor as binding of chemokines does not result in mobilization of intracellular calcium, nor does it provoke direct chemotaxis of CXCR7-positive cells.[9,10] Conversely, CXCR7 is involved in various biologic processes including tumor formation,[8,11] transendothelial migration of tumor cells,[12] modulation of CXCL12-induced migration,[9] internalization of the ligand after chemokine exposure,[9] angiogenesis,[13] and recruitment of renal stem cells.[14]

At the moment relatively little is known about the expression of CXCR7 in human tissues. CXCR7 was described on normal as well as neoplastic endothelial cells,[8,15,16] and proliferation of these cells was prevented by antisense molecules to CXCR7.[17] There has been some controversy on the expression of CXCR7 on inflammatory cells, such as monocytes, B cells, and dendritic cells.[18,19] Chemotaxis of T cells toward CXCL12 was blocked by an anti-CXCR7 antibody, whereas other groups did not reproduce these results.[8,9] In gene chip analysis of human immune cells, the expression of CXCR7 was shown on B cells, some T-cell subsets, and NK cells, but there was only a marginal effect of CXCR7 deficiency on B-cell development in mice.[20]

CXCR7 is an interesting molecule for allograft injury, as both of its ligands were found to be of functional importance in allograft models, and are present in human allograft tissue.[21–25] As an essential prerequisite for studies on the functional role of CXCR7, we evaluated the expression of CXCR7 and localized it in human renal allografts.

RESULTS

Expression of CXCR7, CXCL11, and CXCL12 mRNA in renal allograft biopsies

The mRNA expression of CXCR7 and the corresponding ligands was studied in microdissected tissues from allograft biopsies of patients with borderline lesions ($n=12$), acute rejection ($n=23$; Banff I, 10; Banff II, 13), signs of chronic injury ($n=10$), and biopsies taken before implantation from living donors ($n=8$) as controls (Figure 1, Table 1). All three genes were found to be expressed in the tubulointerstitium of renal allografts (Figure 1). CXCL11 mRNA showed the strongest expression, significantly increased during acute rejection, and was significantly lower in patients with signs of chronic injury (as compared to acute rejection, Figure 1a). CXCL12 mRNA was also found to be induced during acute allograft rejection (Figure 1b). Interestingly, both chemokines were significantly increased in biopsies with borderline lesions, with no difference between biopsies with borderline lesions and biopsies with acute rejection (Figure 1a and b). CXCR7 mRNA was detected in all biopsies but showed no change of expression levels in rejection or chronic injury (Figure 1c). A positive correlation was found between the expression of CXCL12 and the expression of CXCR7 (Spearman $r=0.53$, $P<0.0001$), but not with the expression of CXCL11 (not significant). There was no correlation between CXCR7, CXCL12, CXCL11, and serum-creatinine or proteinuria at the time of biopsy. CXCR7 was not consistently detected by real-time RT-PCR (40 cycles) in isolated glomeruli of the same patient cohort.

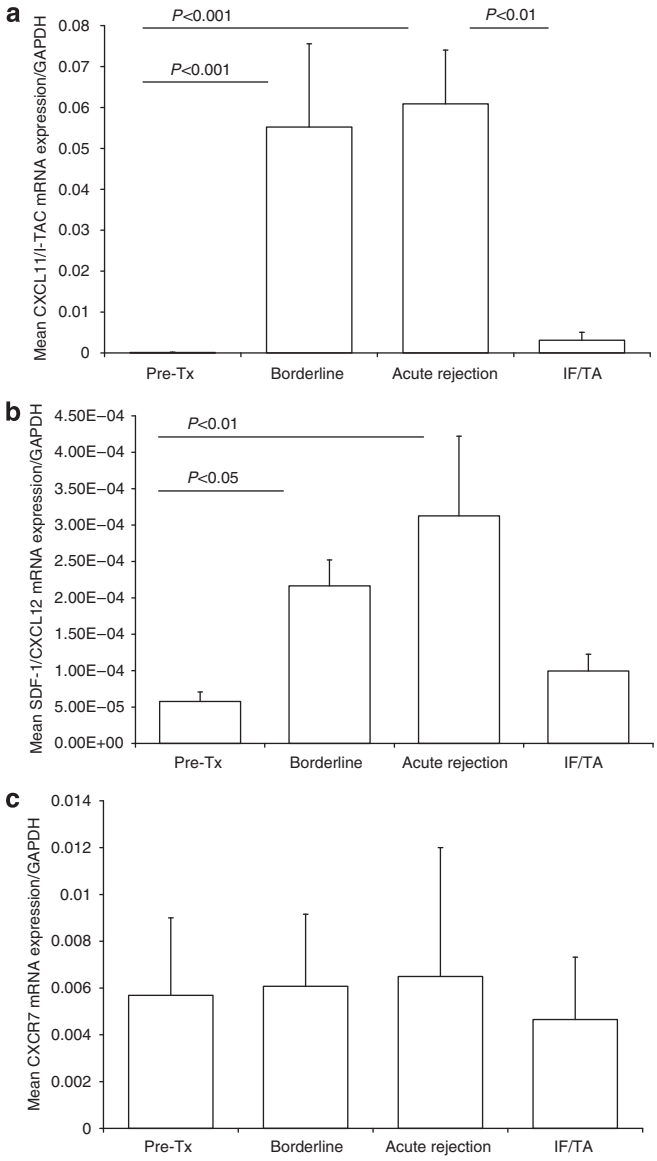

Figure 1 | mRNA expression of CXCL11, CXCL12, and CXCR7 in the tubulointerstitium. The mRNA expression was quantified by real-time RT-PCR in renal allograft biopsies with borderline lesions (borderline), acute rejection, signs of chronic injury (IF/TA), and from living donors taken before implantation as controls (pre-Tx). (**a** and **b**) A significant induction of CXCL11 (**a**) and CXCL12 (**b**) mRNAs was present in biopsies with borderline lesions and with acute rejection. (**c**) No change in CXCR7 mRNA expression was detectable.

Localization of CXCR7 in well-preserved renal tissue from tumor nephrectomies and pretransplant biopsies

Immunohistochemistry for CXCR7 was performed in well-preserved renal tissue reflected by 12 biopsies from allografts taken before implantation and the normal area of a tumor nephrectomy specimen (Figure 2). In these well-preserved kidneys, three sites of CXCR7 protein expression were identified, that is, endothelial cells, smooth muscle cells of vessel walls, and tubular epithelial cells (Figure 2a–c). The endothelial cells of scattered peritubular capillaries were CXCR7 positive, but the overall number of CXCR7-positive vessels was low (Figure 2a). Some larger veins showed CXCR7-positive endothelial cells (Figure 2e). No CXCR7-positive cells were found to be present in the normal glomerular tufts (Figure 2b and d). The arterioles at the vascular pole stained positive for CXCR7 (Figure 2d). Smooth muscle cells of arterial walls showed a positive signal (Figure 2b and e). Some tubular epithelial cells were positive on the basolateral side (Figure 2c). Morphologically, the CXCR7-positive tubular segments were consistent with distal tubules and collecting ducts.

Expression of CXCR7 in renal allografts

To describe the expression of CXCR7 in different forms of renal allograft injury, we stained sections from allograft nephrectomies due to terminal failure (Figure 3), as well as renal allograft biopsies, including biopsies with acute rejection (Banff I, $n=22$; Banff II, $n=11$; antibody mediated, $n=13$) and with chronic allograft injury ($n=6$). The basic clinical parameters were summarized in Table 2.

Table 1 | Basic clinical data on the mRNA study population (range)

Diagnosis	*n*	Gender (m/f)	Mean age (years)	Time after Tx (months)	Creatinine (mg/dl)
Borderline	12	9/3	49 (25–66)	4.7 (0–24)	3.9 (1.5–10.4)
AR	23	17/6	52 (38–71)	3.3 (0–24)	5.7 (2–10.2)
IF/TA	10	NA	59 (42–71)	89 (12–180)	3.0 (1.4–6.2)
Pre-Tx	8	NA	50 (27–63)	NA	<1.1

Abbreviations: AR, acute rejection; f, female; IF/TA, interstitial fibrosis/tubular atrophy; m, male; NA, not available; Pre-Tx, biopsy taken before implantation of the allograft; Tx, transplantation.

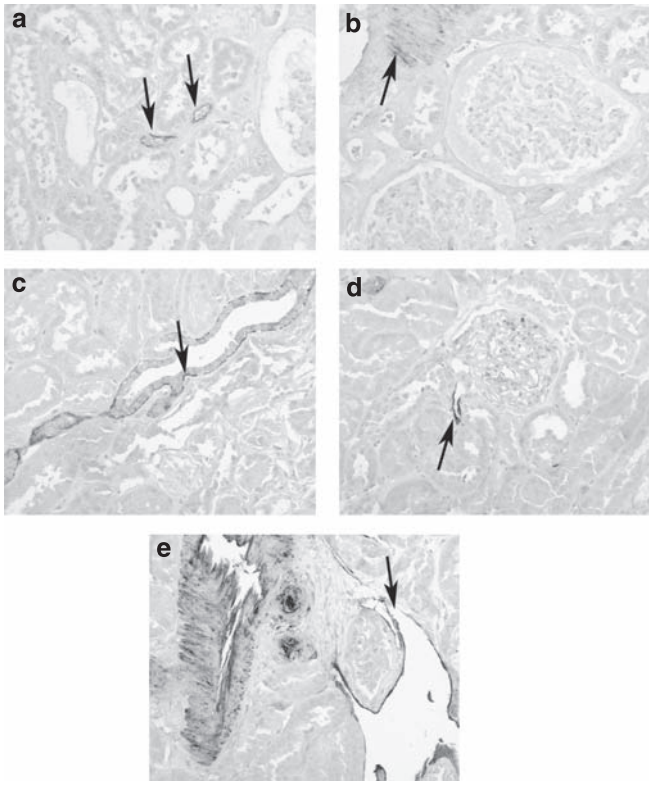

Figure 2 | Expression of CXCR7 in controls. Immunohistochemistry was performed on sections from tumor nephrectomies (**a**, **b**) and pretransplant biopsies (**c**–**e**) with a monoclonal antibody against CXCR7 (original magnification × 200). Expression of CXCR7 was detected on endothelial cells (**a**, **d**, **e**, arrows), on smooth muscle cells of arteries (**b**, arrow), and the basal aspect of tubular epithelial cells (**c**, arrow). Please note the low number of peritubular capillaries positive for CXCR7 (**a**).

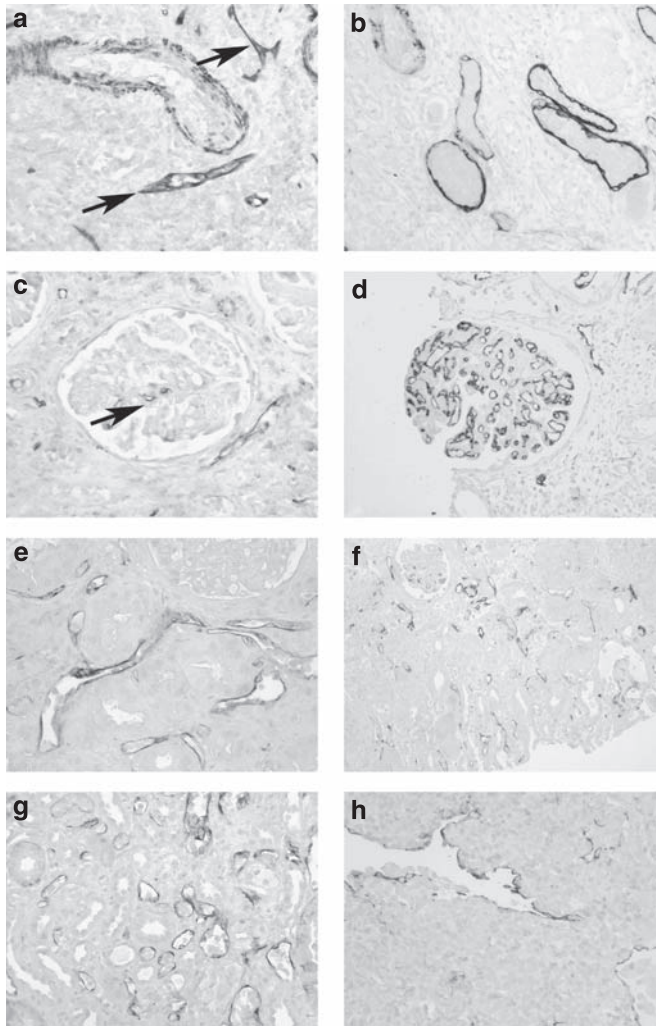

Figure 3 | CXCR7 expression in allograft nephrectomies and renal biopsies with acute allograft rejection. Immunohistochemistry was performed on sections of allograft nephrectomies (**a**–**d**) or allograft biopsies with acute interstitial rejection (Banff I, **e**), acute vascular rejection (Banff II, **f**), and acute antibody-mediated rejection (**g**, **h**) with a monoclonal antibody against CXCR7 (original magnification × 200 **a**–**d**, **g**; × 250 **e**; × 100 **f**–**h**). A prominent number of CXCR7-positive vessels (**a**, arrow) were present in the tubulointerstitium. Glomerular capillaries were only occasionally CXCR7 positive (ranging from single capillary loops (**c**, arrow) to the whole glomerular tuft (**d**) being positive for CXCR7).

A prominent number of CXCR7-positive small vessels in the tubulointerstitium was present in renal allografts (Figure 3). This high number of CXCR7-positive interstitial vessels was found in all forms of acute allograft rejection (Figure 3e–h). CXCR7-positive vessels in the tubulointerstitium were associated with sites of inflammatory cell accumulation (Figure 3a and h). It was common that CXCR7-positive vessels were found within or in proximity to sites of nodular infiltrates (Figure 3h). Morphologically, two types of interstitial vessels were present. The first resembled peritubular capillaries (Figure 3g). The second type was present in proximity to arteries, but also in the rest of the cortex with a slit-like appearance (Figure 3a and e). Morphology and distribution of these vessels were consistent with lymphatic vessels. Although the overall number of these CXCR7-positive vessels was higher than in controls, there was a high variability between biopsies with the same morphological diagnosis. Interestingly, the biopsies with acute humoral rejection showed the lowest number of CXCR7-positive vessels. There was no association with the depositon of C4d. Furthermore, there was no association between the number of CXCR7-positive vessels and serum-creatinine (at the time of biopsy), or the number of globally sclerosed glomeruli (indicating progressive injury).

Table 2 | Basic clinical data on the immunohistochemistry study population (range)

Diagnosis	n	Gender (m/f)	Mean age (years)	Time after Tx (months)	Creatinine (mg/dl)
Banff I	22	13/9	52 (27–71)	5.5 (0.2–28.3)	2.6 (0.9–5.2)
Banff II	11	4/7	53 (21–68)	0.9 (0.2–3.7)	4.3 (1.2–8.0)
AMR	13	8/5	44 (26–71)	4.8 (0.2–28.6)	4.6 (2.0–7.1)
IF/TA	6	5/1	59 (39–75)	82 (4.4–195)	2.5 (1.8–3.0)
Pre-Tx	12	6/6	50 (30–75)	—	NA

Abbreviations: AMR, antibody-mediated rejection; f, female; IF/TA, interstitial fibrosis/tubular atrophy; m, male; NA, not available; Pre-Tx, biopsy taken before implantation of the allograft; Tx, transplantation.

Table 3 | Basic clinical data on the protocol biopsies (range)

Diagnosis	n	Gender (m/f)	Mean age (years)	Time after Tx (months)	Creatinine (mg/dl)
No R	12	8/4	48 (25–62)	3.8 (0.3–10)	1.5 (0.9–2)
AR	7	5/2	46 (35–67)	3.2 (0.2–6)	2.4 (1.2–3.3)*
IF/TA	5	4/1	49 (32–67)	3.5 (3–4)	1.9 (1.5–3)

Abbreviations: AR, acute rejection; f, female; IF/TA, interstitial fibrosis/tubular atrophy; m, male; R, rejection; Tx, transplantation.
*$P<0.05$ vs no R.

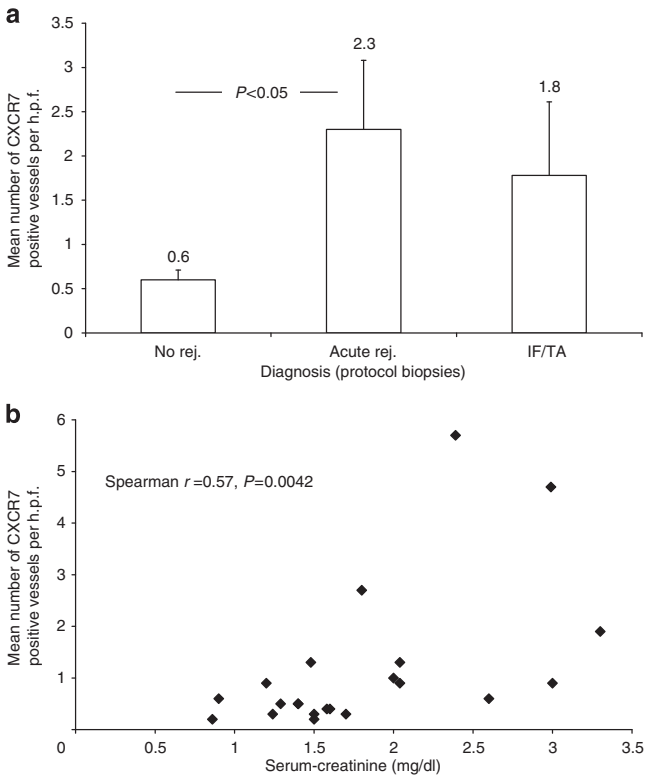

Figure 4 | Quantification of CXCR7 positive vessels in protocol biopsies. (**a**) Illustrated are the mean numbers of CXCR7 positive vessels per high power field (h.p.f.), in biopsies with no rejection (no rej.), with acute rejection (acute rej.), and with signs of chronic injury (IF/TA). The number of CXCR7 positive vessels was significantly increased in biopsies with acute rejection. (**b**) A significant correlation was documented between serum-creatinine and the number of CXCR7 positive vessels.

Glomerular expression of CXCR7 on capillaries of the tuft was found in allografts, but not in normal tissue (Figures 2, 3c and d). Expression on the endothelium of the glomerular tuft was variable and ranged from a low number of glomerular capillary loops (segmental expression) to CXCR7 expression on the majority of glomerular capillaries (Figure 3c and d). Although the expression of CXCR7 was most prominent on glomeruli with signs of chronic allograft glomerulopathy, it was not regularly associated with this condition. In the allograft nephrectomy and transplant biopsies, the CXCR7 expression on smooth muscle cells and tubular epithelial cells was present as described for the normal controls (Figure 3a).

Expression of CXCR7 on protocol biopsies

To confirm the results in an independent group of biopsies, we performed immunohistochemistry for CXCR7 on an additional series of 24 protocol biopsies. These biopsies showed no signs of rejection ($n=12$), acute rejection (borderline, $n=4$; Banff I, $n=2$; Banff II, $n=1$), or chronic lesions with interstitial fibrosis and tubular atrophy ($n=5$). In the group with chronic lesions, two patients additionally showed borderline lesions. The clinical data are presented in Table 3. The vast majority of protocol biopsies were performed at 3–4 months after transplantation.

The distribution of CXCR7 mirrored the pattern in controls and indication biopsies. In the 12 biopsies without signs of rejection, the number of CXCR7-positive vessels was low (Figure 4a). A higher number of positive vessels was present in protocol biopsies that showed acute rejections (Figure 4a, $P<0.05$). The number of CXCR7-positive vessels in patients with chronic lesions was higher than in controls, but the difference did not quite reach the level of significance (Figure 4a). The expression of CXCR7 on smooth muscle cells of arteries and in some tubular epithelial cells did not differ from the other morphological entities. The number of CXCR7-positive vessels was not associated with the number of globally sclerosed glomeruli. The serum-creatinine at the time of biopsy correlated with the number of CXCR7-positive interstitial vessels in this population (Figure 4b).

Expression of CXCR7 on lymphatic endothelium

The localization of CXCR7-positive small vessels close to larger arteries, with a 'slit' appearance, indicated that these might be lymphatic vessels. Therefore, we performed multicolor immunofluorescence for podoplanin (a marker of lymphatic vessels) and CXCR7 (Figures 5 and 6). In the tubulointerstitium, CXCR7-positive vessels were present, which were also positive for podoplanin (Figure 5). An additional population of CXCR7-positive small vessels was podoplanin negative (that is, blood vessels). Both types expressed the endothelial cell marker CD34 (not shown).

The number of single- and double-positive vessels was quantified per high power field (Figure 6). In pretransplant biopsies a low number of CXCR7-positive vessels was present (Figure 6), with nine times more CXCR7-positive vessels than

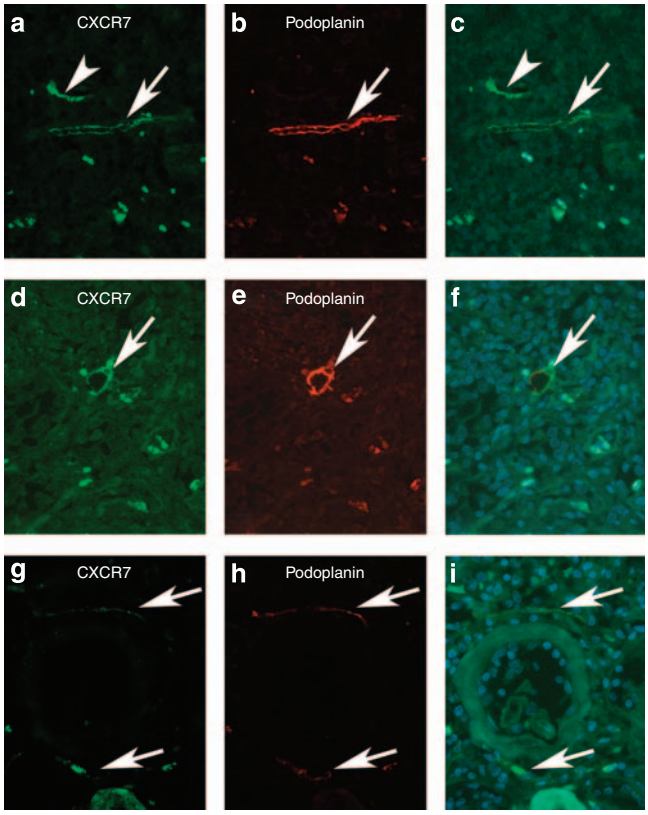

Figure 5 | Localization of CXCR7 on lymphatic endothelium. Multicolor immunofluorescence for CXCR7 (**a**, **d**, **g**) and lymphatic endothelium (podoplanin, D2-40, **b**, **e**, **h**) was performed on tonsil (**a**-**c**) or renal allograft nephrectomies (**d**-**i**, original magnification $\times$ 400). Nuclei are counterstained in blue (**c**, **f**, **i**). The overlay is shown in **c**, **f**, **i**. The arrows localize double positive (CXCR7- and podoplanin-positive lymphatic vessels), the arrowheads show a CXCR7-positive vessel, which is podoplanin negative (that is, a blood vessel).

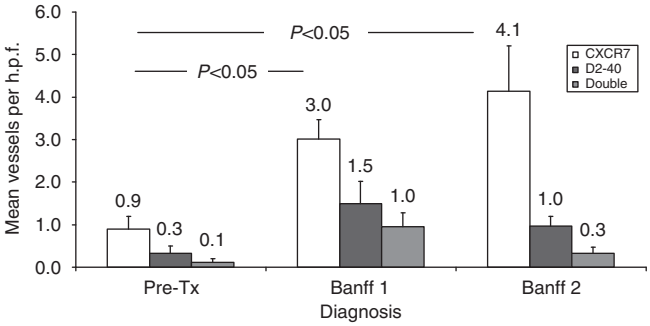

Figure 6 | Quantification of the multicolor immunofluorescence. Multicolor fluorescence for CXCR7 and the lymphatic marker podoplanin (D2-40) was performed and the positive vessels were quantified (CXCR7, D2-40, and double positive). There was a significant increase of CXCR7-positive vessels in biopsies with acute rejection.

CXCR7-positive lymphatic vessels. In acute cellular rejection, the number of CXCR7-positive vessels was significantly higher (Banff I) as compared to biopsies taken before implantation ($P<0.05$, Figure 6). The ratio between CXCR7-positive vessels and lymphatic vessels was 3:1 (whereas it was 9:1 in normal tissue). This indicates an absolute increase in CXCR7-positive lymphatic vessels. Two-third of the lymphatics were CXCR7 positive (whereas in normal tissue only one-third of the lymphatics was CXCR7 positive).

In vascular rejection, the number of CXCR7-positive vessels was also significantly higher compared to biopsies taken before implantation ($P<0.05$, Figure 6). The ratio of CXCR7-positive lymphatic vessels and blood vessels remained similar to that seen in the biopsies taken before implantation. Therefore, although the absolute numbers of both vessel types increased, the main rise in CXCR7-positive vessels was due to the single-positive peritubular capillaries (blood vessel endothelium).

DISCUSSION

CXCR7 is a chemokine receptor that recently received a lot of attention.[7,11,12,14] It shares one ligand with the receptor CXCR4 (CXCL12) and one with CXCR3 (CXCL11). Both of these receptors were previously described in human renal allografts, but were mainly found to be expressed by infiltrating cells. In this study, we describe the expression of CXCR7 on microvascular endothelial cells, with an induction during acute allograft rejection in human renal allografts.

Also various functions of CXCR7, an atypical chemokine receptor, have been recently described. Zabel *et al.*[12] showed that ligand binding to CXCR7 led to the recruitment of β-arrestin2. Either CXCL11 or a small molecule CXCR7 ligand blocked transendothelial migration of tumor cells (which were CXCR4 and CXCR7 positive).[12] For renal stem cells, it has also been shown that blocking either CXCR4 or CXCR7 reduced the engraftment to injured kidneys and transendothelial migration also required both receptors.[14] Heterodimers form between CXCR7 and CXCR4, which impairs CXCL12-mediated responses in primary lymphocytes.[26] For these functions, CXCR7 and CXCR4 need to be present on the same cell, which might be important for stem cells, tumor cells, and inflammatory cells.

In line with our results, CXCR7 has also been shown to be present on endothelial cells. Expression of CXCR7 was induced by pro-inflammatory cytokines in human umbilical vein endothelial cells *in vitro*.[8] Hypoxia induced CXCR7 on microvascular endothelial cells.[27] Induction of CXCR7 was also found on endothelial cells transformed by Kaposi's sarcoma-associated herpesvirus,[15] and proliferation of these cells was prevented by antisense molecules to CXCR7.[17] Melanoma and malignant brain tumors were described to contain CXCR7-positive vessels.[16,27] The adhesion of cells to human umbilical endothelial cells was increased by endothelial CXCR7 expression.[8] Therefore, CXCR7 is expressed on a subset of blood endothelial cells, where it is upregulated under inflammatory and hypoxic conditions. This is consistent with our results of CXCR7 expression by these cells in an inflammatory state such as acute renal allograft rejection.

It might be important that in zebrafish the migration and correct localization of CXCR4-positive primordial germ cells was dependent on the expression of CXCR7-positive somatic cells.[28] CXCR7 knockdown resulted in misguidance of the primordial germ cells.[28,29] In this setting, the scavenging of CXCL12 paves the way for new chemotactic gradients. The function of endothelial CXCR7 in allografts remains to be defined. It could increase adhesion (as shown for umbilical vein endothelial cells), it could induce chemokine release (as shown for CXCL8 in prostate carcinoma cells[30]), and it might sharpen gradients. Whether CXCR7 might increase the availability of CXCL11 and CXCL12 on the apical site of endothelial cells (as it has been described for the Duffy antigen/receptor for chemokines, another chemokine binding protein) has not yet been shown.[31]

Lymphatic endothelial cells are a site of expression that have not been shown previously. Studies on lymphatic vessels and particularly their function in renal allografts are rapidly evolving.[32–34] The number of lymphatic vessels has been shown to increase in renal allografts in association with sites of inflammation and chronic injury.[32,33] In other organs, such as the transplanted heart, lymphatic vessels have been shown to be of recipient origin.[35] Accumulation of inflammatory cells within these newly formed lymphatic vessels might indicate a functional role in transplant rejection.[32–34] The impact of these vessels on allograft survival is under intensive debate, with the current hypotheses ranging from a potential positive to a detrimental effect. In a study on renal allograft protocol biopsies, the graft function at 1 year after transplantation was better when lymph vessels were present at sites of inflammation compared to biopsies without lymphatics in areas of cell recruitment.[35] Here we show the expression of CXCR7 on lymphatic endothelial cells in renal allografts. The number of CXCR7-positive vessels increased during acute interstitial allograft rejection. Therefore CXCR7 is strategically situated at the site of inflammatory cell efflux on lymphatics to modulate the chemokine gradients at this site. The exit of inflammatory cells, particularly dendritic cells, is regulated in part through chemokines. Particularly the CCR7 ligand CCL21, and the CXCR7 ligand CXCL12 have been shown to be involved in lymphatic trafficking.[36] The functional role on lymphatic endothelial cells in renal allografts will undoubtedly be an important question for future studies.

We found that both known ligands of CXCR7, that is, CXCL11 and CXCL12, were significantly induced in human renal allografts with acute allograft rejection. CXCL11 has been previously localized in rat heart allografts to infiltrating macrophages and endothelial cells.[37] CXCL12 expression was also found to be of functional importance in a rat kidney transplantation model, as an anti-CXCL12 antibody delayed signs of chronic rat allograft injury.[21] The strong induction of CXCL11 during acute rejection has been previously shown by Panzer *et al.*[38] Using *in situ* hybridization, a diffuse expression of CXCL11 in the tubulointerstitium, most likely on endothelial cells was demonstrated.[38] CXCL11 has also been described to be elevated in urine of patients with renal allografts.[25] Interestingly, we found both chemokines to be significantly upregulated in borderline lesions, with the level of expression not significantly different to acute rejection. It was previously thought that these chemokines function through the classical receptors CXCR3 and CXCR4.[38,39] We add a new receptor with CXCR7, which might modulate the function of these chemokines in renal allografts. In contrast to the increased number of CXCR7-positive vessels by immunohistochemistry, we were unable to detect an induction of CXCR7 mRNA. Differences between mRNA expression data and localization of the protein can be related to the absence of mRNA regulation, posttranslational regulation differences.[8] Furthermore, it would seem that the induction of CXCR7 on endothelial cells represents a very small number of cells within a large volume of the kidney. The changes in the signal of CXCR7 mRNA in the vessels might not be detectable due to the background noise of a variety of signals from other cells in the tubulointerstitial space. Hence, although the change on the vessels is significant, the change in the signal for mRNA derived from the whole tubulointerstitial space might not be detectable.

We were unable to show a direct correlation between renal function and the number of CXCR7-positive vessels in indication biopsies, but this association was significant in protocol biopsies. Importantly, CXCR7-positive vessels were also associated with sites of inflammatory cell accumulation. From the increased expression of CXCR7 in association with inflammatory cell accumulation, it might be hypothesized that CXCR7 might be involved in adhesion of cells to peritubular capillaries and lymphatic vessels.

In summary, CXCR7 on endothelial cells might modulate the recruitment of inflammatory cells and the activation of endothelial cells in renal allografts. These hypotheses generated with the help of our new morphological findings should now be tested in functional studies. CXCR7-deficient mice die shortly after birth due to heart malformations, which makes allograft models in these mice impossible.[20] Therefore, CXCR7 blocking agents will have to be used to further define the biological function of CXCR7 in allograft injury.

MATERIALS AND METHODS

Study population for the localization of CXCR7 by immunohistochemistry

Renal allograft biopsies were formalin-fixed and paraffin-embedded following routine protocols (the morphological parameters are summarized in Table 2). Archival sections were used in this study. Included were a total of 64 indication allograft biopsies with acute interstitial rejection (Banff I, $n=22$), acute vascular rejection (Banff II, $n=11$), allograft biopsies with signs of chronic injury ($n=6$), C4d-positive biopsies with either no signs of cellular rejection ($n=8$) or additional signs of cellular rejection ($n=5$). Biopsies taken before allograft implantation served as controls ($n=12$). An additional series of 24 protocol biopsies were included. These biopsies included 12 without significant lesions, 7 with acute forms of rejection (borderline, $n=4$; Banff I, $n=2$; Banff 2, $n=$ II), and 5 with chronic lesions with interstitial fibrosis and tubular atrophy. CXCR7 was localized in all specimens by immunohistochemistry.

Real-time RT-PCR

To quantify the mRNA expression of CXCR7, CXCL11, and CXCL12, we used real-time RT-PCR as described.[40] The biopsy samples used for the mRNA analysis were from a different cohort than the morphological study. Clinical data are summarized in Table 1. The renal biopsies were obtained from a multicenter renal biopsy bank (the European Renal cDNA Bank–Kroener-Fresenius biopsy bank). Informed consent was obtained before renal biopsies were performed. Microdissected tubulointerstitial compartments were from a total of 53 biopsies with borderline lesions ($n=12$), acute rejection ($n=23$, Banff 97 I, 10; Banff 97 II, 13), signs of chronic injury (interstitial fibrosis and tubular atrophy, $n=10$), and biopsies taken before implantation from living donors as controls ($n=8$).

Reverse-transcription and real-time RT-PCR was performed as reported earlier.[41] Pre-developed TaqMan reagents were used for the human target genes CXCR7, CXCL11, and CXCL12 (all from Applied Biosystems, Darmstadt, Germany), and two endogenous control genes (18S rRNA and GAPDH; Applied Biosystems). The expression of target genes was normalized by both reference genes, 18S rRNA and hGAPDH, which resulted in comparable results. Data shown are normalized to GAPDH. The mRNA expression was analyzed by standard curve quantification. All measurements were performed in duplicates. Controls consisting of double distilled water were negative in all runs.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[2] In brief, dewaxed and rehydrated tissue sections were incubated in 3% hydrogen peroxide (to block endogenous peroxidases). 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). The primary antibodies were applied for 1 h. Incubation with biotinylated secondary antibodies (Vector) for 30 min was followed by the ABC reagent (Vector). 3′3′-Diaminobenzidine (Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) was used as a detection system. As the primary antibody a monoclonal mouse anti-human CXCR7 (provided by ChemoCentryx, Mountain View, CA, USA) was used. This antibody has been previously described in detail.[8,42]

Establishment of the antibody

Human tonsils and allograft nephrectomies were used to establish the staining protocol. A heat-based antigen retrieval (using an autoclave oven) resulted in a very reliable staining pattern (Figure 7). The isotype IgG controls did not show black color product (Figure 7a). CXCR7 was predominantly expressed on endothelial cells of small vessels and capillaries, and larger veins showed CXCR7-positive endothelial cells (Figure 7). High endothelial venules and leukocytes were CXCR7 negative. The basal epithelial layer was found to be CXCR7 positive.

Immunofluorescence

Multicolor immunofluorescence was performed for CXCR7 and podoplanin (a marker of lymphatic endothelium) on all indication biopsies. The monoclonal antibody D2-40 was used to detect podoplanin and was visualized with a Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). CXCR7 was visualized by a biotinylated secondary and labeled with FITC bound to streptavidin (Vector). After the first antigen was labeled, another microwave treatment was performed to omit cross-reaction of secondary antibodies. Negative controls consisted of isotype immunoglobulins for both primary antibodies.

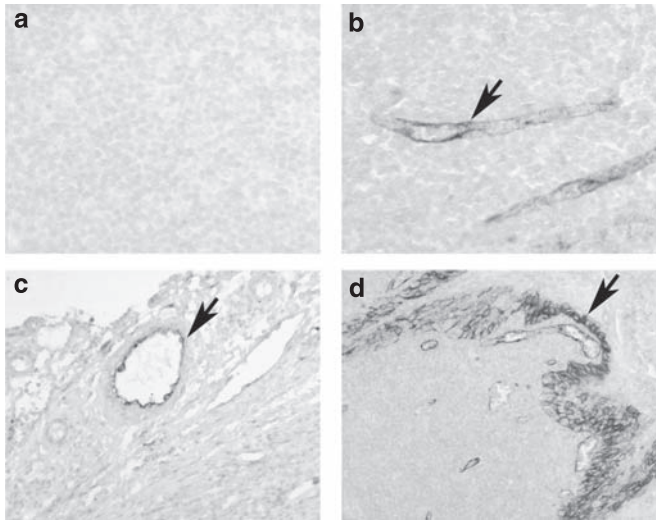

Figure 7 | Immunohistochemistry for CXCR7 on human tonsils. Immunohistochemistry was performed on tissue sections from human tonsils with an isotype control antibody (**a**) or a monoclonal antibody against human CXCR7 (**b**–**d**; original magnification $\times 400$ **a**, **b**; original magnification $\times 200$ **c**–**d**). No positive color product was found in isotype controls (**a**). Endothelial cells of small vessels (**b**, arrow) stained positive for CXCR7. The endothelium of arteries and larger veins only occasionally showed positive staining (**c**, arrow). Furthermore, the basal epithelial layer was CXCR7 positive (**d**, arrow).

Statistics and quantification of results

The number of CXCR7-positive vessels, podoplanin-positive lymphatics, and double-positive lymphatics were counted per high power field (original magnification $\times 400$) by two observers masked to the diagnosis. The mean of the two quantifications is shown.

For the protocol biopsies, 10 digital pictures (original magnification $\times 250$) were taken, and the number of positive vessels was evaluated by an observer masked to the diagnosis. The nonparametric Kruskal–Wallis test was used to compare the mean number of positive vessels. A value of $P<0.05$ was considered to be significant. Error bars show standard error of the mean (s.e.m).

DISCLOSURE

Mark Penfold and Thomas Schall are employees of ChemoCentryx. All the other authors declared no competing interests.

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