Characterization of CXCL16 and ADAM10 in the normal and transplanted kidney

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The chemokine CXCL16 plays an important role in the recruitment of leukocytes to sites of inflammation influencing the course of experimental glomerulonephritis. Here we show that human kidneys highly express CXCL16 in the distal tubule, connecting tubule and principal cells of the collecting duct with weak expression in the thick ascending limb of Henle. Beside the membrane localization, a soluble form of CXCL16 can be proteolytically released which acts as a chemotactic factor. In human renal tissue the expression pattern of the disintegrin-like metalloproteinase ADAM10 is similar to that of CXCL16, suggesting ADAM10 can potentially cleave CXCL16 in vivo. When we tested this in primary tubular cells we found that blockade of ADAM10 activity inhibited the IFN-γ induced release of soluble CXCL16. Acute tubular damage in renal allografts was associated with elevated urinary CXCL16 and this correlated with focally increased apical CXCL16 expression in the distal tubules and collecting ducts. Renal allograft biopsies, with a histopathological diagnosis of acute interstitial rejection, showed increased basolateral ADAM10 expression together with high numbers of infiltrating T cells. Our results suggest that CXCL16 and ADAM10 are involved in the recruitment of T cells to the kidney and play an important role in inflammatory kidney diseases.

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The infiltration of leukocytes into sites of tissue damage is a characteristic feature of an inflammatory process. It is well established that chemokines are critical for this recruitment of specific leukocyte subsets.[1] The majority of chemokines are secreted as small, soluble molecules. In contrast to these secretory molecules, two chemokines, CXCL16 and fractalkine/CX3CL1, are anchored in the cell membrane.[2,3] Recently an important role of CXCL16 in the development of kidney diseases has been described. For instance, blocking of CXCL16 by specific antibodies ameliorated experimental immune nephritis in mice.[4] In addition, CXCL16 played a major role in the acute inflammatory or progressive phase of established glomerulonephritis in rats.[5] Blocking antibodies against CXCL16 significantly attenuated monocyte/macrophage infiltration and glomerular injury in an anti-glomerular basement membrane model.[5]

As a surface-expressed molecule, CXCL16 can function as a scavenger receptor for phosphatidylserine and oxidized lipoproteins and, thereby, enhance the uptake of oxidized low-density lipoproteins.[6] Beside this, CXCL16 can also exist as a soluble molecule, inducing chemotaxis of CXCR6-expressing T, natural killer, and natural killer T cells to sites of inflammation and injury.[7] Soluble CXCL16 is generated from the cell surface by a process called ectodomain shedding. The most prevalent protease for the constitutive CXCL16 shedding is the disintegrin-like metalloproteinase ADAM10.[8,9]

Very few data exist about the expression of CXCL16 and ADAM10 in the human kidney and their role in renal disease progression. In this study, we have analyzed the expression of ADAM10 and CXCL16 in the normal human kidney. We demonstrate that ADAM10 and CXCL16 are constitutively expressed and colocalize in renal tubular cells of distal convoluted tubule (DCT), the connecting tubule (CNT), and in cells of the collecting duct (CD). In addition, using a specific metalloproteinase inhibitor and small interfering

RNA (siRNA) experiments against ADAM10, we present evidences that ADAM10 is involved in the interferon-γ (IFN-γ)-induced cleavage of CXCL16 in Thick Ascending Limb of Henle's Loop and Early Distal Tubular Cells (TALDCs). Notably, targeting ADAM10 and CXCL16 with siRNAs in primary TALDC reduced chemotaxis of CXCR6-positive T cells, suggesting that ADAM10 and CXCL16 are involved in the recruitment of immune cells in inflammatory processes in the kidney. Furthermore, we were able to detect elevated levels of urinary CXCL16 in kidney allograft recipients mirrored by focally increased apical expression of CXCL16 in the distal tubules and CDs of allograft biopsies. Additionally, in biopsies of patients with diagnosis of acute interstitial rejection, we found enhanced basolateral ADAM10 expression in distal renal tubuli, which was associated with an increased infiltration of T cells. In summary, our data suggest that ADAM10 and CXCL16 may play important roles during the process of renal inflammatory diseases such as acute allograft rejection and may be useful targets for therapeutic intervention.

RESULTS

CXCL16 is mainly expressed in segment-specific cells of DCT, CNT and CD of normal human kidney

To investigate the *in vivo* expression of CXCL16 in the human kidney, we analyzed serial sections of normal renal tissue by immunohistochemistry. As shown in Figure 1a, in the renal cortex and outer stripe, constitutive expression of CXCL16

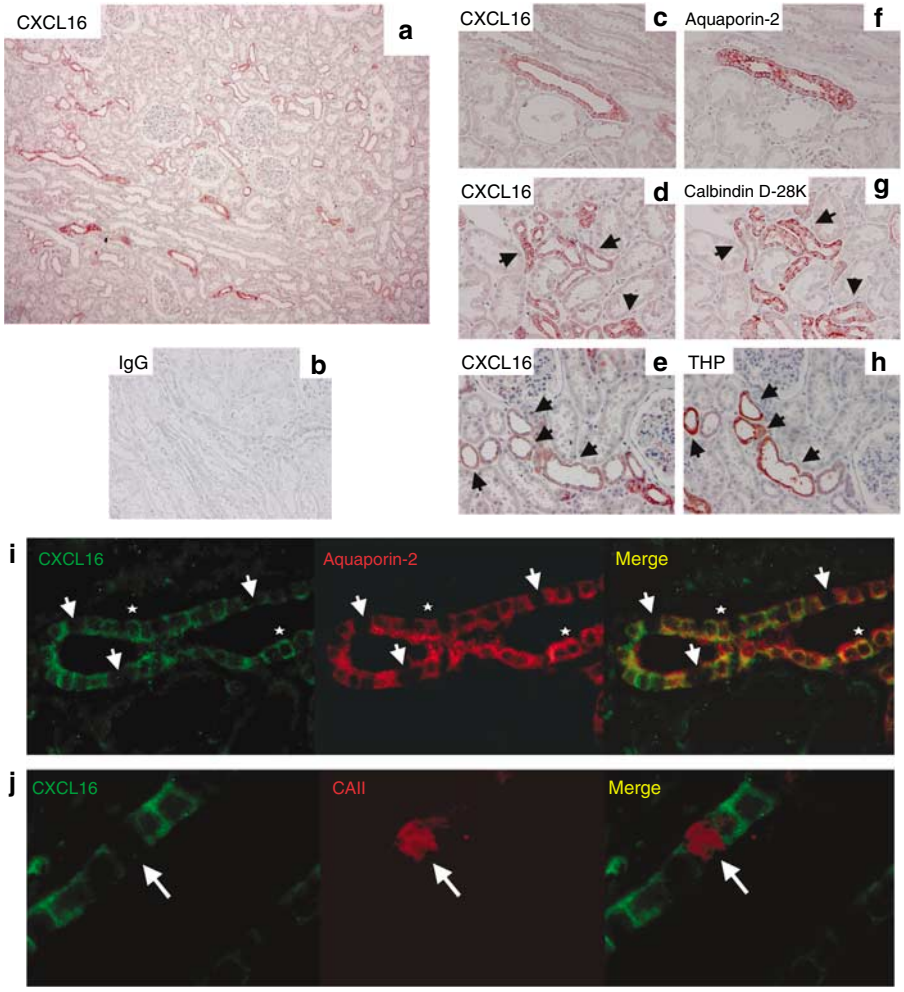

Figure 1 | CXCL16 localization in human kidney sections of healthy parts of tumor nephrectomies. (**a**) In the overview, CXCL16 protein is constitutively expressed in tubular cells of the healthy kidney. Paraffin-embedded sections of normal human renal tissue were stained with a polyclonal antibody against CXCL16. (**b**) Renal tissue stained with rabbit isotype IgG antibody was negative. Immunohistochemical analysis of serial paraffin-embedded sections of normal renal tissue revealed its expression in the kidney cortex in the CD (**c**); in distal convoluted tubules, connecting tubules, and the initial parts of cortical CDs (**d**), and in the TAL of Henle's loop (**e**), as shown by colocalization of CXCL16 with aquaporin-2 (**f**), calbindin D-28K (**g**), and Tamm Horsfall glycoprotein (**h**), respectively, in the same nephron segments (examples are indicated by arrows). Note, that the proximal tubule was devoid of any signal for CXCL16. (**i**) Double immunofluorescent staining of CXCL16 and aquaporin-2 in a cortical CD. CXCL16 expression (green) is detected in many aquaporin-2-positive (red) principal cells of the CD; examples are marked with asterisks. In contrast, arrows indicate cells negative for either aquaporin-2 or CXCL16, thus representing presumably intercalated cells. (**j**) CXCL16 expression is not present in the intercalated cells. Double staining with antibodies against carbonic anhydrase II (CAII, a marker for intercalated cells, red fluorescence) and CXCL16 (green fluorescence) demonstrates that intercalated cells (arrows) do not express CXCL16, since both signals do not colocalize.

was found in tubular cells. Weak expression of CXCL16 was also seen in the glomeruli of the kidney. To confirm the specificity of CXCL16 immunostaining, we used a rabbit isotype control antibody, which did not show any immunoreactivity (Figure 1b). Using antibodies against aquaporin-2 (a marker protein for the CD), calbindin D-28K (a marker protein for distal convoluted tubules, connecting tubules and early parts of cortical CDs), and Tamm–Horsfall Glycoprotein, expressed in the TAL, we can show that CXCL16 was constitutively expressed in the CD (Figure 1c and f), in distal convoluted cells, in connecting tubules (Figure 1d and g), and a weak expression of CXCL16 was also found in the cortical TAL cells (Figure 1e and h). No CXCL16 expression was found in the proximal tubules. In the outer medulla, CXCL16 was expressed in CDs but not in TAL cells. In detail, we identified the segment-specific cells of DCT, CNT, and CD as the CXCL16-expressing cells. Double immunofluorescence using antibodies against aquaporin-2 (as a marker for principal cells in the CD) and CXCL16 demonstrated CXCL16 expression in the principal cells of the CD (Figure 1i). Vice versa, no CXCL16 expression was seen in intercalated cells, as shown with double staining experiments with anti-carbonic anhydrase II and CXCL16 antibodies (Figure 1j).

ADAM10 is involved in the IFN-γ induced release of CXCL16 in primary TALDC

It is known that transmembrane CXCL16 can be cleaved by ADAM10, and the soluble form of CXCL16 can act as a chemoattractant for immune cells.[9] To characterize the metalloproteinase responsible for the shedding of CXCL16, we analyzed the supernatants of primary human renal TALDCs using a specific CXCL16 enzyme-linked immunosorbent assay (ELISA). As shown in Figure 2a, IFN-γ induced approximately a twofold increase in the release of CXCL16. The ADAM10-specific metalloproteinase inhibitor, GI254023X, and the broad-spectrum metalloproteinase inhibitors, GM6001 and TAPI-2, blocked the IFN-γ-stimulated release of CXCL16 (Figure 2a). To confirm this result, we also analyzed cell-surface-bound CXCL16. As expected, inhibition of metalloproteinases increased the level of cellular CXCL16 as shown by western blot analysis (Figure 2b) and CXCL16-specific ELISA (Figure 2d). With siRNA against CXCL16, both bands were significantly reduced, verifying the specificity of the CXCL16 antibody used in our western blot analysis. Furthermore, using ADAM10 siRNA, we were able to very efficiently knock down the expression of ADAM10 protein in primary TALDCs (Figure 2e). The amount of soluble CXCL16 was significantly decreased in the supernatant of ADAM10 siRNA-treated cells (Figure 2f), thus confirming that ADAM10 is involved in the IFN-γ-stimulated cleavage of CXCL16 in TALDCs.

ADAM10 is constitutively expressed in the segment-specific cells of the DCT, CNT, and CD in the human kidney

To prove that ADAM10 may also be the potential protease cleaving CXCL16 in tubular cells *in vivo*, we analyzed the expression of ADAM10 in the human kidney. In the renal cortex, ADAM10 was constitutively expressed in tubular profiles, and weakly in scattered glomerular cells (Figure 3a). Constitutive ADAM10 expression could be observed in the DCT and CNT (Figure 3c and f), CD (Figure 3b and e), and a weaker expression was found in cells of the cortical TAL (Figure 3d and g). However, the expression level seemed to be roughly stronger than it was for CXCL16 in the hitherto mentioned nephron segment. To again separate intercalated cells from segment-specific cells, double labeling with specific antibodies for detection of principal (aquaporin-2) and intercalated cells (H^+-ATPase) were performed. This allowed us to identify principal cells as ADAM10-expressing cells in the CD (Figure 3h). Intercalated cells did not express ADAM10 (Figure 3i). Importantly, confocal analysis revealed that ADAM10 and CXCL16 are constitutively expressed in the same tubular cells (Figure 3j).

Blocking soluble CXCL16 with siRNAs against ADAM10 and CXCL16 decreases the chemotaxis of CXCR6-expressing T cells

The recruitment of leukocytes into the kidney has emerged as a key event in the development of inflammatory kidney diseases.[10] The only known receptor of CXCL16 is CXCR6. CXCR6 is expressed on CD4-positive effector memory T-cell subsets and natural killer T cells.[11,12] Using fluorescence-activated cell sorting analysis, we confirmed that Jurkat T cells express transmembrane CXCR6 (Figure 4a). Using siRNAs against CXCL16 and ADAM10 we could reduce the release of CXCL16 from tubular cells (Figure 4b). In IFN-γ-treated tubular cells, ADAM10 inhibition reduced the amount of soluble CXCL16 to almost constitutive levels. In contrast, CXCL16 knockdown completely abolished the release of CXCL16 (Figure 4b). Soluble CXCL16 is known to recruit CXCR6-expressing cells to sites of inflammation.[12] To investigate the role of soluble CXCL16 released by TALDCs in this recruitment process, we performed chemotaxis assays using supernatants from siRNA-transfected TALDCs (Figure 4b). As shown in Figure 4c, employment of supernatants of TALDC-treated with CXCL16 and ADAM10 siRNA reduced the chemotaxis of T cells. Note, the strongest effect was seen with supernatants of ADAM10 siRNA-treated TALDCs.

Differential expression of CXCL16 and ADAM10 in transplanted kidneys with ATN and IR and determination of urinary CXCL16 levels

To investigate the involvement of ADAM10 and CXCL16 in the recruitment of T cells in biopsies of kidneys of transplanted patients, we analyzed in a pilot study the distribution of ADAM10 and CXCL16 in 11 kidney transplanted patients diagnosed with acute interstitial rejection (IR) or acute renal tubular damage as controls with double immunofluorescence. IR served as an example for inflammatory processes in the human kidney, where infiltration of T cells is the major component.[13]

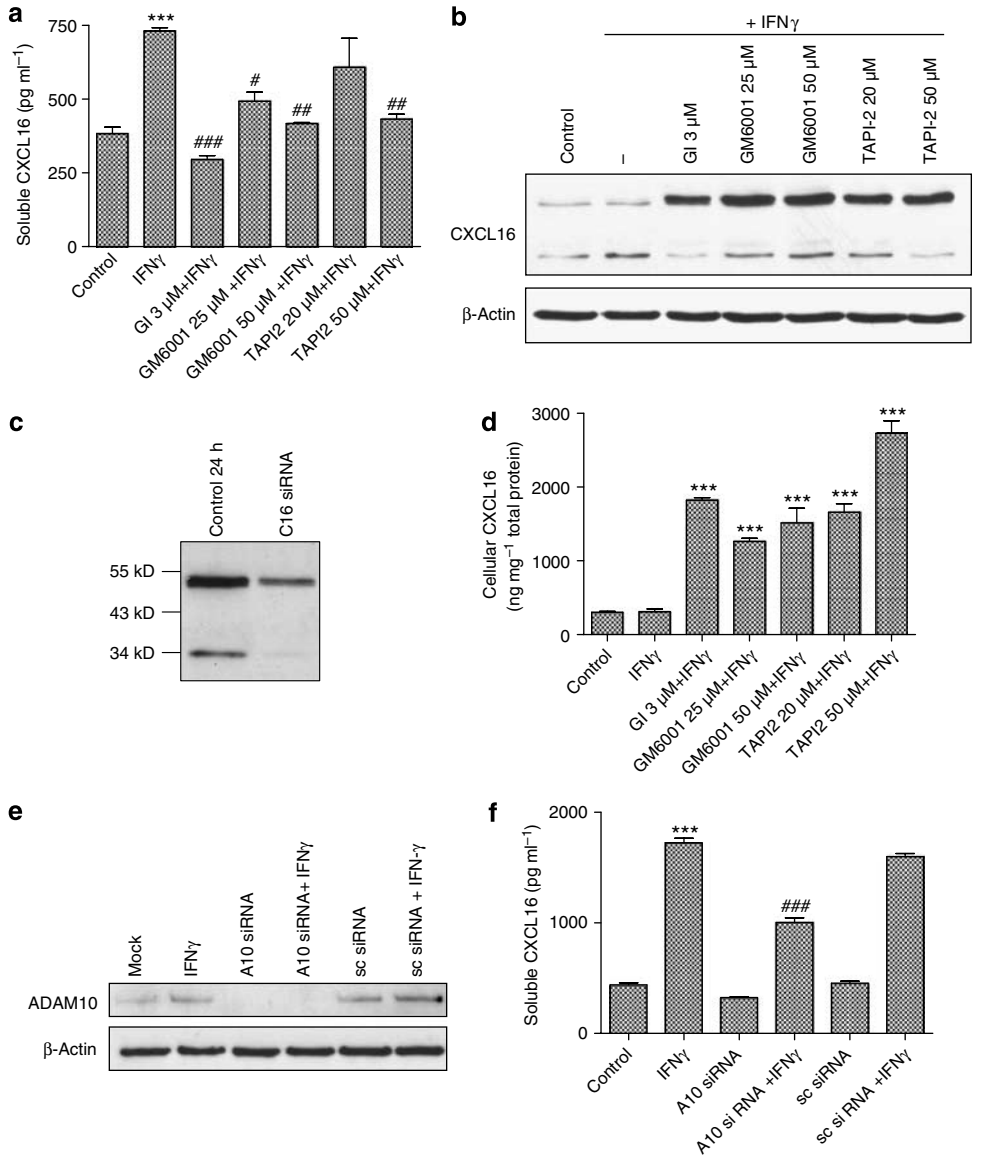

Figure 2 | Influence of metalloproteinases on the expression and release of CXCL16. (**a**) Analysis of soluble CXCL16 released from primary TALDCs with a CXCL16-specific ELISA. Cells were preincubated with different proteinase inhibitors for 15 min before IFN-γ (24 ng/ml) was added for 24 h. Application of IFN-γ (24 ng/ml) increased the amount of soluble CXCL16 in the supernatants of TALDCs. The ADAM10-specific metalloproteinase inhibitor, GI254023X (GI), and the broad-spectrum metalloproteinase inhibitors, GM6001 and TAPI-2, reduced the IFN-γ-induced CXCL16 release. Data are mean ± s.d. ($n = 3$); $^{***}P < 0.001$ versus control; $^{###}P < 0.001$, $^{##}P < 0.01$; $^{#}P < 0.05$ versus cytokine-treated cells. (**b**) Inhibition of metalloproteinases increased cellular CXCL16 protein expression in TALDCs. Fifteen minutes before application of IFN-γ, cells were pretreated with the indicated concentration of the metalloproteinase inhibitors GI254023X, GM6001, and TAPI-2. CXCL16 protein levels were measured by western blot analysis and β-actin was used as a loading control. (**c**) Knockdown of CXCL16 with CXCL16-specific siRNA in TALDCs is shown by western blot analysis. (**d**) Application of pharmacological inhibitors of metalloproteinases increased the amount of cellular CXCL16. Cell lysates were isolated as described under Materials and Methods, and 15 μg total proteins were used to measure the amount of cell-expressed CXCL16 by CXCL16-specific ELISA. Data are mean + s.d. ($n = 5$). Statistically significant release of CXCL16 ($P < 0.001$) is indicated by asterisk. (**e**) Suppression of ADAM10 protein levels by RNA interference in the presence and absence of IFN-γ. Cell lysates of TALDCs were prepared 48 h after transfection with siRNA specific for ADAM10 and an unspecific siRNA. Mock-transfected cells treated with the transfection lipid in the absence of siRNA were used as a control. Western blot analysis of ADAM10 protein expression was performed. Blots were reprobed with an antibody specific for β-actin as a loading control. (**f**) Inhibition of ADAM10 by siRNA reduced the IFN-γ-stimulated CXCL16 release in TALDCs. Supernatants of siRNA-treated and IFN-γ-stimulated or unstimulated cells were analyzed for soluble CXCL16 using a CXCL16-specific ELISA ($n = 3$); $^{***}P < 0.001$ versus control; $^{###}P < 0.001$ versus IFNγ-treated cells.

The clinical characteristics together with CXCL16 and ADAM10 expression pattern of the patient's biopsies are summarized in Tables 1 and 2. In all acute tubular necrosis (ATN) patients, after renal transplantation, expression of CXCL16 was increased in a focal manner on the apical side of distinct tubuli in comparison with normal kidneys (Figure 5a and b), whereas ADAM10 expression was not changed significantly (Figure 5b). The strong apical expression of

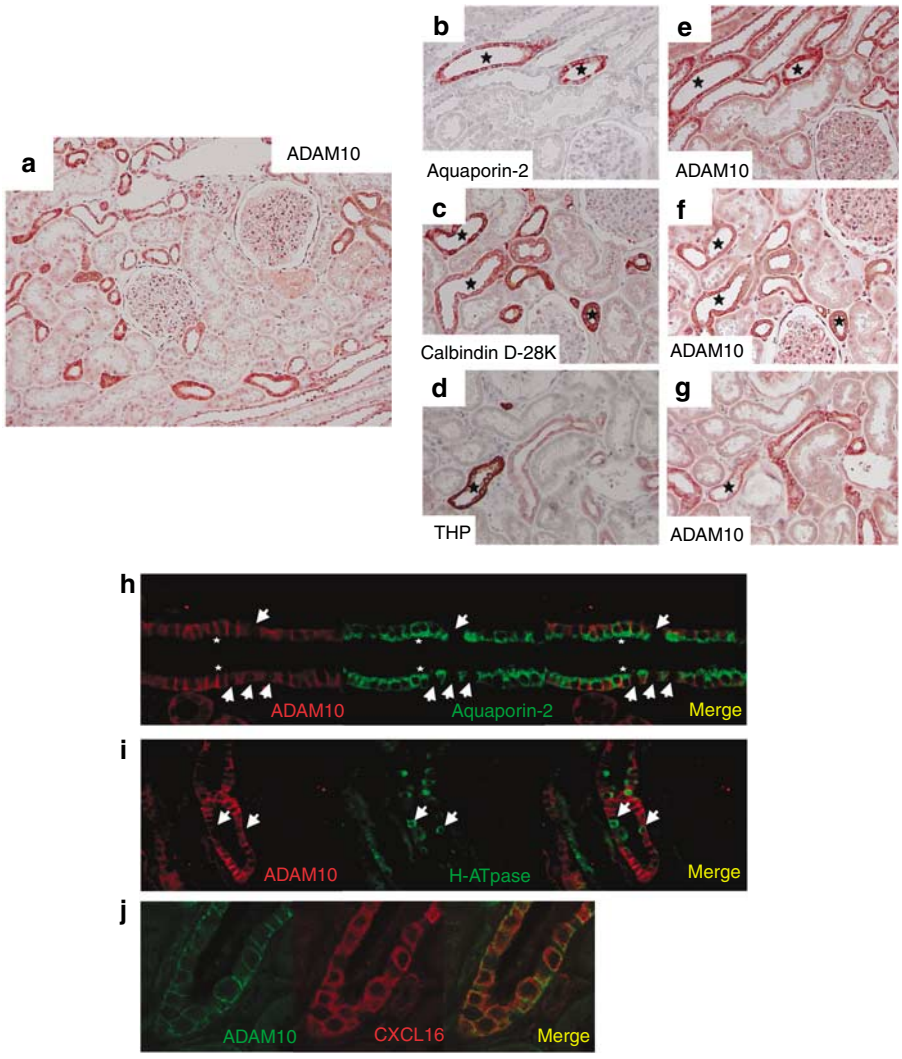

Figure 3 | Localization of ADAM10 protein in normal human kidney. (**a**) In the overview, ADAM10 protein is mainly expressed in tubular cells, but expression was also found in glomerular cells. Serial sections of normal renal tissue represented constitutive ADAM10 expression in aquaporin-2 (**e**), calbindin D-28K (**f**), and Tamm–Horsfall glycoprotein (**g**) expressing tubular profiles, which correspond to CD, (DCT, CNT), and TAL, respectively. Proximal tubules were devoid of any specific ADAM10 staining. (**h**) Immunofluorescent detection of ADAM10 (red fluorescence) in aquaporin-2-positive principal cells of the CD (green fluorescence). A prominent overlap of both signals in the same cells can be seen, indicated in the examples by asterisks. In contrast, arrows indicate cells negative for either aquaporin-2 or ADAM10, thus representing presumable intercalated cells. (**i**) ADAM10 is not expressed in the intercalated cells of the CD. Note: arrows represent H^+-ATPase-expressing intercalated cells (green colour) that do not express ADAM10 (red colour); compare with the merged view. (**j**) Confocal immunofluorescence analysis of ADAM10 and CXCL16 expression in a cortical CD. Tissue section was stained with Alexa Fluor 488 and Cy3 secondary antibodies to visualize the localization of CXCL16 (red) and ADAM10 (green) proteins, respectively. Both signals can be confined to same segment-specific cells; see merged picture on the right.

CXCL16 correlated with elevated urinary CXCL16 levels (Table 1). In contrast, in healthy volunteers only weak amounts of urinary CXCL16 could be detected (Figure 5c).

In addition, compared with normal renal tissue and ATN, we detected stronger distal tubular ADAM10 expression in all allograft biopsies with the diagnosis of IR (Figure 6; Table 2). Importantly, in one patient (Figure 6, patient B1), who had the highest level of infiltrating T cells (Table 2), very strong tubular ADAM10 (Figure 6, arrows) expression was observed along with decreased or no CXCL16 expression in the same tubuli (Figure 6, arrows). Interestingly, in some distal tubuli of another patient (Figure 6, patient B5) strong expression and colocalization of ADAM10 and CXCL16 could be detected (Figure 6, patient B5). In summary, in all kidney transplant patients with IR, increased tubular ADAM10 expression and variable CXCL16 expression could be observed, which was paralleled by high levels of infiltrating T cells. Moreover, less pronounced amounts of urinary CXCL16 were measured at the time of IR (Figure 5c).

DISCUSSION

The migration of leukocytes through vessels and beyond the vascular compartment is dependent in part on small chemoattractant proteins called chemokines. Leukocyte

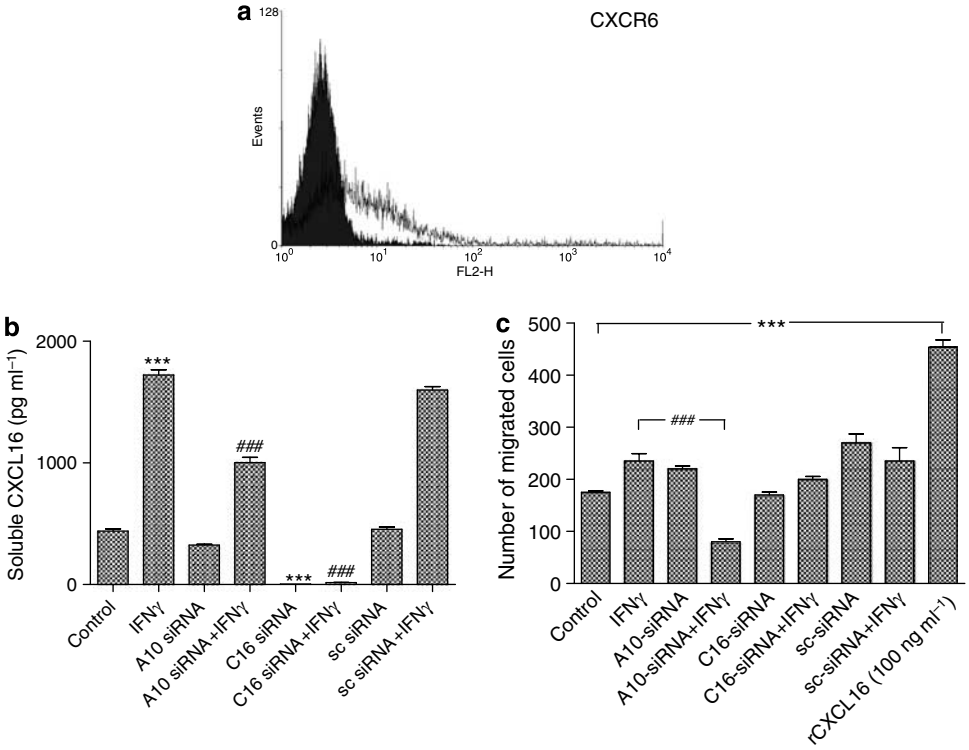

Figure 4 | Supernatants from ADAM10- and CXCL16 siRNA-transfected TALDCs decreased the chemotaxis of Jurkat T cells. (**a**) Surface expression of the chemokine receptor CXCR6 in Jurkat T cells analyzed by fluorescence-activated cell sorting analysis. (**b**) Inhibition of CXCL16 protein expression by siRNA fully abolished release of CXCL16 in TALDCs. Cells were transfected with different siRNA duplexes for inhibition of ADAM10 or CXCL16, an unspecific siRNA (scrambled), or not transfected with siRNA (mock). A total of 24 h after transfection, TALDCs were stimulated with IFN-γ for 24 h, where indicated, and supernatants were collected. Soluble CXCL16 was determined by a CXCL16-specific ELISA ($n = 4$). Data are mean ± s.d.; $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus IFN-γ-treated cells. (**c**) Decrease of soluble CXCL16 correlates with a reduction in the migration of Jurkat T cells. Supernatants shown in Figure 5b were used for chemotaxis assays ($n = 2$). Recombinant CXCL16 induced migration of CXCR6-expressing Jurkat T cells about threefold; $^{**}P < 0.01$; $^{*}P < 0.05$ versus control.

Table 1 | Clinical features and CXCL16 and ADAM10 analysis in ATN patients with renal biopsy

Case	Gender	Age	Days after TX	Serum creatinine (mg/dl)	Creatinine clearance (ml/min/1.73 m^2)	Urinary CXCL16 (pg/ml)	T cells Tub. Int. cells/HPF	Focally apical tubular CXCL16 expression	Basolateral tubular ADAM10 expression
A1	M	52	21	1.65	44	651	23.8	+++	+
A2	F	65	25	3.7	33	441	18.1	+++	+
A3	F	61	29	3.1	15.3	468	26.8	++	++
A4	M	65	15	2.9	21.9	1,209	20.1	+++	+
A5	M	62	33	2.5	39.7	428	5.2	+++	+
A6	M	27	12	6.0	11.3	122	8.4	++	+

+, intensity of CXCL16 or ADAM10 expression in comparison with that in normal kidney; ATN, acute tubular necrosis; HPF, high-power field; Tub. Int., tubular interstitium; TX, transplantation.

Table 2 | Clinical features and CXCL16 and ADAM10 analysis in IR patients with renal biopsy

Case	Gender	Age	Days after TX	Serum creatinine (mg/dl)	Creatinine clearance (ml/min/1.73 m^2)	Urinary CXCL16 (pg/ml)	T cells Tub. Int. cells/HPF	Mean tubular CXCL16 expression	Basolateral tubular ADAM10 expression
B1	M	63	90	2.15	31.2	137	131.3	–	+++
B2	M	34	32	1.62	49	91	78.1	–	+++
B3	M	45	36	3.7	17.9	473	62.2	–	++
B4	M	71	22	3.2	19.2	91	73.8	–	++
B5	F	43	20	2.1	23.1	444	59.2	++	+++

–, decreased expression of CXCL16 as compared with normal CXCL16 expression; +, intensity of ADAM10 expression in comparison with that in normal kidney; HPF, high-power field; IR, interstitial rejection; Tub. Int., tubular interstitium; TX, transplantation.

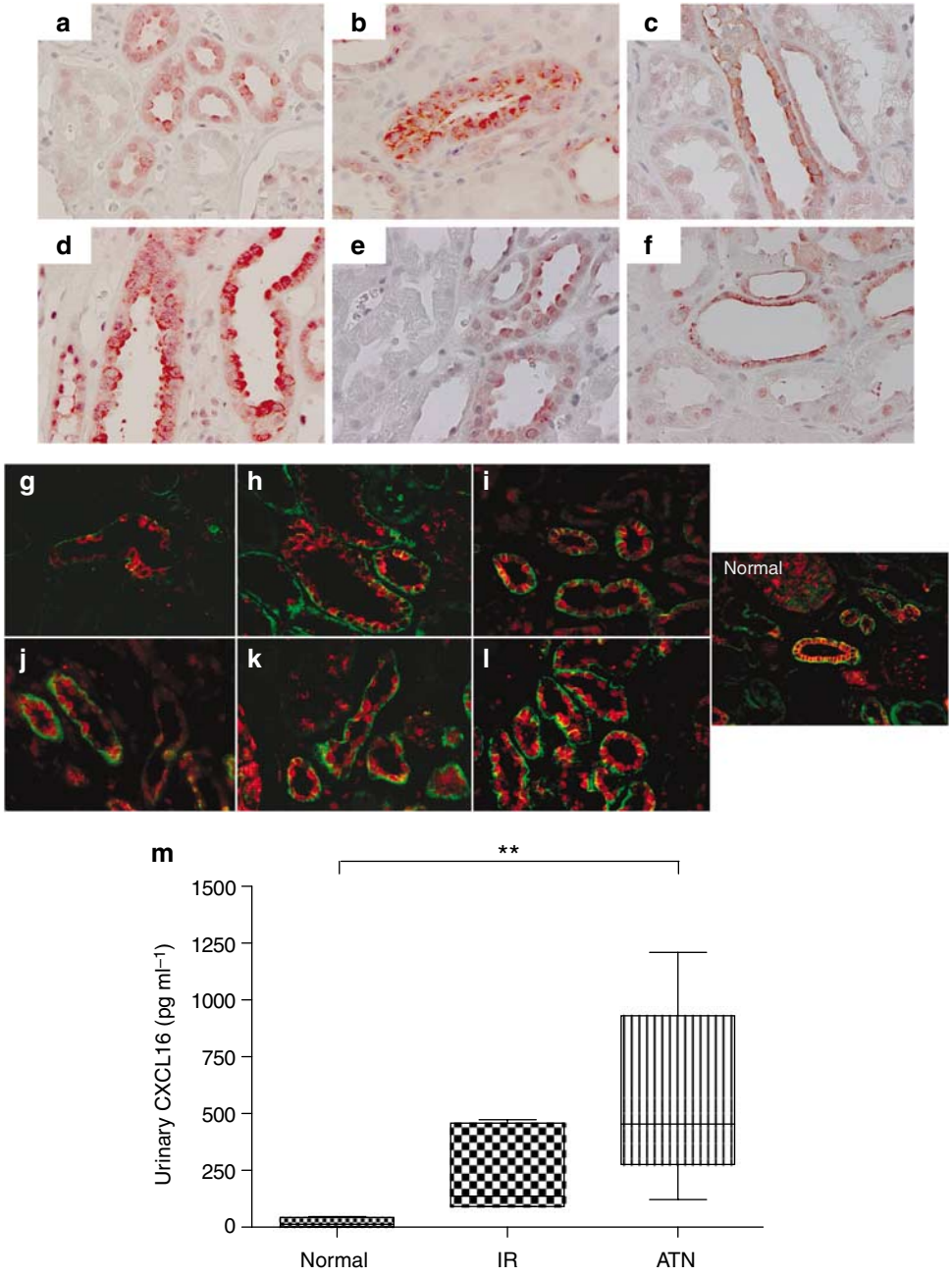

Figure 5 | Increased urinary CXCL16 correlated with focally apical CXCL16 expression in tubular cells of renal allografts with the histopathological diagnosis of ATN. Six allograft biopsies of patients diagnosed with ATN (cases A1–A6; clinical parameters and CXCL16 analysis are shown in Table 1) were analyzed by CXCL16 immunohistochemical analysis (**a**–**f**) and with ADAM10 (green colour) and CXCL16 (red colour) double immunofluorescence staining (**g**–**l**). Notably, all patients showed focally increased apical CXCL16 expression in renal tubuli. Strongest apical CXCL16, seen in patient A4 (**d**), correlated with the highest amount of urinary CXCL16 (Table 1). In contrast, ADAM10 expression (green colour) was not significantly changed in ATN patients (A1–A6) compared with normal kidney (**g**–**l**). (**m**) Urinary CXCL16 measured by a CXCL16-specific ELISA in healthy volunteers (normal), in patients with IR, and in patients with ATN. Data are mean ± s.d.; $^{**}P < 0.01$ versus control.

traffick from the peripheral blood into the tubulointerstitial compartment of the kidney is a hallmark of almost any kind of renal inflammatory disease. Interestingly, some chemokines are known to be expressed constitutively, whereas proinflammatory chemokines are exclusively expressed in response to specific stimuli. For instance, the transmembrane chemokine, CXCL16, is known to be expressed in epithelial cells, like keratinocytes, in the absence of inflammatory activators.[14] In this study, we could show, for the first time, constitutive expression of CXCL16 in distal tubular cells in normal human renal tissue. In a mouse model of experimental immune nephritis, increased CXCL16 expression was

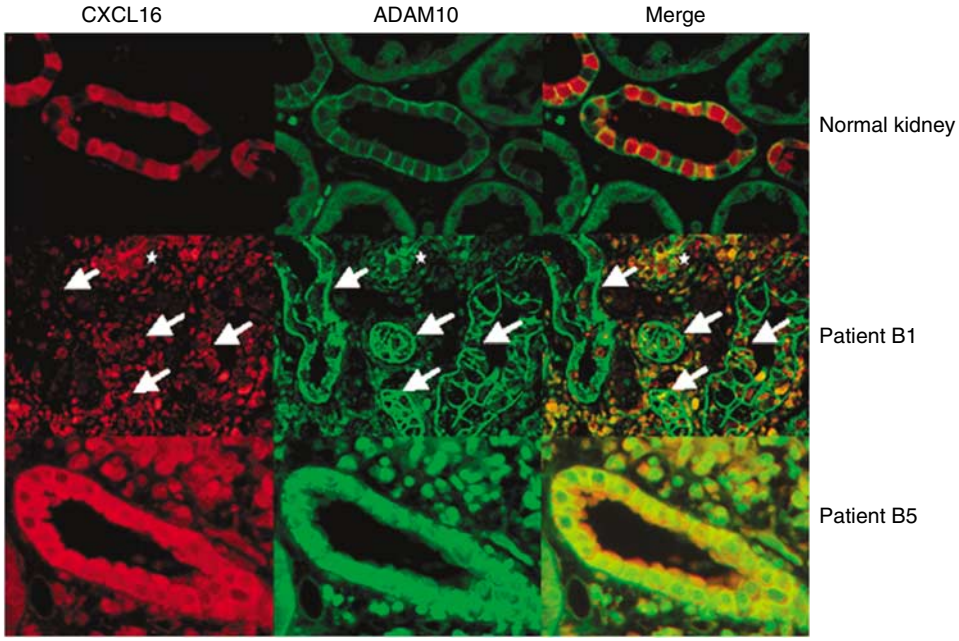

Figure 6 | Increased ADAM10 expression in allograft biopsies of kidney transplant patient with the clinical and histopathological diagnosis of acute IR. Renal allograft biopsies of patients diagnosed with IR were analyzed by double immunofluorescence for ADAM10 and CXCL16 expression in comparison with normal kidney (upper panel). Clinical features and ADAM10 analysis of patients are listed in Table 2. Tissue sections were stained with Alexa Fluor 488 and Cy3 secondary antibodies to visualize the localization of CXCL16 (red) and ADAM10 (green) proteins, respectively. In some tubuli of patient B1 (marked with arrows), strong basolateral ADAM10 expression and no CXCL16 expression could be seen (middle panel). In contrast, in few tubuli of the same patient (marked with a star) both molecules colocalized. In the lower panel (patient B5), strong coexpression of ADAM10 and CXCL16 in a tubule is visible, accompanied with interstitial inflammatory infiltrates.

found in the glomeruli, tubules, and in the vascular endothelium.[4] *In vitro* studies identified lipopolysaccharide-stimulated mesangial cells as a rich source of the chemokine CXCL16.[4] Our immunohistochemical analysis of normal human renal tissue identified tubular cells as the main CXCL16-expressing cells. Specifically, CXCL16 expression was identified in segment-specific cells of the DCT, CNT, cortical CD, and in the TAL, but not in intercalated cells. Different hypotheses can be presented concerning the role of the constitutive expression of CXCL16 in tubular cells. Membrane-bound CXCL16 is known to mediate adhesion and phagocytosis of Gram-negative and Gram-positive bacteria. Notably, CXCL16 is not only able to trigger phagocytosis of bacteria, but can also directly kill bacteria.[15] In humans, membrane-bound CXCL16 is expressed on macrophages, monocyte-derived dendritic cells, and blood myeloid dendritic cell.[16] Dendritic cells can release CXCL16, which acts as a chemoattractant molecule and recruits CXCR6-expressing cells, like activated T cells and natural killer cells, to sites of inflammation. In this study, we were able to show that primary TALDC increased levels of soluble CXCL16 after IFN-γ treatment. Therefore, also in the kidney, this increased release of CXCL16 could play an important role in the recruitment of T cells during inflammation to sites of kidney injury.

In our study, we could show that in primary TALDCs ADAM10 is involved in processing of CXCL16. A specific ADAM10 metalloproteinase inhibitor, as well as siRNA against ADAM10, efficiently blocked the IFN-γ-induced cleavage of CXCL16 in TALDCs. Interestingly, in fibroblasts and endothelial cells, ADAM10 has been implicated in constitutive shedding of CXCL16,[8,9] whereas ADAM17 seems to be involved in the phorbol-ester-induced shedding of the chemokine. Using primary TALDCs, we could demonstrate that IFN-γ induced a more than fourfold increase in the shedding of CXCL16. Only ADAM10 siRNA could significantly inhibit the IFN-γ-induced CXCL16 shedding in TALDC, whereas ADAM17 siRNA had only a marginal effect (data not shown). Furthermore our study provides *in vivo* evidence that ADAM10 is localized like CXCL16 in the segment-specific cells of the distal nephron and CD, but not in intercalated cells, presuming that ADAM10 is also *in vivo* the proteinase cleaving CXCL16.

It is well established that soluble CXCL16 acts as a chemoattractant for CXCR6-expressing immune cells.[3,12] With CXCL16- and ADAM10-knockdown experiments in TALDCs, we were able to underline the importance of ADAM10 and soluble CXCL16 in the recruitment of T cells. Importantly, in renal allograft biopsies of patients with acute interstitial rejection, we found increased tubular ADAM10 expression, which correlated with high numbers of infiltrating T cells. Together with our ADAM10-knockdown experiment in TALDCs, which reduced the chemotaxis of T cells below basal levels, we can speculate that ADAM10, beside

CXCL16, is an attractive new target molecule for treatment of renal inflammatory processes. Of note, in biopsy-proven IR, increased ADAM10 expression (Figure 6, arrows) was encountered with variable CXCL16 expression, including decreased, focally absent or increased expression. The absent or decreased tubular CXCL16 expression could be explained by an increased shedding of CXCL16. This notion is supported by two findings: first, we found that inhibition of ADAM10 increased cellular CXCL16 (Figure 2b) in TALDCs. Second, we were not able to detect soluble CXCL16 in western blot analysis using the same CXCL16 antibody used in immunohistochemistry, assuming that this antibody does not detect the cleaved form of CXCL16.

Recently, in patients with lupus nephritis the levels of urinary CXCL16 showed superior specificity and sensitivity in distinguishing subjects with active renal disease.[17] We found elevated levels of urinary CXCL16 in kidney transplant patients with ATN and in patients with IR, who were paralleled by a focally increased apical CXCL16 expression in renal tubuli in ATN patients and an increased basolateral ADAM10 expression of distal tubuli in IR patients. Nevertheless, to determine whether urinary CXCL16 could be a useful biomarker to monitor tubular kidney injury events, ELISA-based studies in larger groups of patients are required.

In summary, we have characterized, for the first time, the *in vivo* expression of ADAM10 and CXCL16 in normal renal tissue. We also have provided preliminary results on the expression of these molecules in renal allograft biopsies of kidney transplant patients with ATN and IR. In IR patients, strong basolateral tubular ADAM10 expression and variable CXCL16 expression accompanied with high numbers of infiltrating T cells was the most interesting finding. In contrast, in ATN patients, basolateral ADAM10 expression was not significantly changed. Therefore, we assume that in IR patients ADAM10 and CXCL16 play an important role in the recruitment of T cells. As we can only speculate that the decreased CXCL16 expression as observed in patient B1 (Figure 6) is due to downregulation of CXCL16 or increased shedding of the chemokine, further analysis in animal models of kidney transplantation should be performed. Using such models, inhibition of CXCL16 and ADAM10 by blocking antibodies and siRNA will provide new data concerning the role of both molecules in rejection processes of transplanted kidneys. Taken this data together, we speculate that targeting of ADAM10 and CXCL16 might offer a new attractive strategy to prevent recruitment of T cells during acute rejection. Future studies will make evident the importance of ADAM10 and CXCL16 in the transplanted human kidney and in inflammatory kidney diseases.

MATERIALS AND METHODS

Kidney sections

Specimens were taken from healthy parts of renal tissue from six different tumor nephrectomies (obtained from two female and four male patients with ages ranging from 34 to 66 years). Kidney tissues were originally submitted for diagnostic purposes and studied in accordance with national and local ethical principles. The use of human tissue samples has been approved by the local ethical committee (ref. no. #11/10/04). Tissue sections of renal allograft biopsies, which were not needed for diagnostic evaluation, were obtained from the Department of Cellular and Molecular Pathology, German Cancer Research Center, Heidelberg, Germany.

Urine samples

Freshly obtained first-morning urine after initial void was collected in a standardized manner from patients after renal transplantation. Immediately, urine samples were spun at 4 °C at 2,000 *g* and three to five aliquots were prepared. After determination of urinary creatinine and total urine protein level, samples were stored at −20 °C until soluble CXCL16 concentrations were detected by ELISA. Urinary CXCL16 of 11 kidney transplant patients were analyzed on the day of biopsy. Creatinine clearance of the patients was calculated on the day of biopsy.

Isolation and culture of human renal tubular epithelial cells

Human renal tubular epithelial cells were separated using antibody-coated magnetic beads as previously described.[18] Primary TALDCs were characterized as previously described[19] and were seeded in six-well plates pre-coated with human collagen IV and fetal calf serum.

siRNA

For downregulation of endogenous CXCL16 and ADAM10 expression, the following siRNA duplexes (MWG Biotech AG, Ebersberg, Germany) were used: CXCL16 construct, 5′-CAUGAAUCGUCU CCGGAAACATT-3′; ADAM10 construct, 5′-AGACAUUAUGAAGG AUUAUTT-3′. As a negative control an unspecific scrambled siRNA duplex (5′-AGGUAGUGUAAUCGCCUUGTT-3′) was used.

Transfection of siRNA

Twenty-four hours before transfection, 5×10^4 cells were seeded in six-well plates. Transfection of siRNA was performed using Oligofectamine (Invitrogen, Karlsruhe, Germany) and 10 nM siRNA duplexes (MWG Biotech AG) per well. Transfection was performed as previously described.[20] All cells were assayed 24–72 h after the transfection procedure. Specific silencing of targeted genes was confirmed by at least two independent experiments using western blot analysis. Conditioned media were harvested and analyzed by ELISA for the presence of released CXCL16.

Cytokines, antibodies, and chemicals

Recombinant human CXCL16, recombinant human IFN-γ, anti-human ADAM10 ectodomain antibody, and anti-human CXCR6 antibody were obtained from R&D Systems (Wiesbaden, Germany). Rabbit CXCL16 (western blot, immunohistochemistry, immunofluorescence) was from Peprotech (London, UK). The rabbit anti-calbindin D-28K (EG-20) and anti-aquaporin-2 antibody (immunohistochemistry and immunofluorescence) were obtained from Sigma (Taufkirchen, Germany). The polyclonal ADAM10 antibody (for immunohistochemistry) was from Chemicon (Hampshire, UK) and the polyclonal ADAM10 antibody (for western blotting) was from Calbiochem (Darmstadt, Germany). The Tamm–Horsfall protein antibody for immunohistochemistry was obtained from BIOTREND GmbH (Cologne, Germany). The H^+-ATPase antibody (sc-20943) and the carbonic anhydrase II antibody (sc-48351) were from Santa Cruz Biotechnology (Heidelberg, Germany). The monoclonal ADAM10 (mAb 11G2, for double

immunofluorescence) to the ADAM10 ectodomain was from Diaclone (Besancon, France). The ADAM10-specific metalloproteinase inhibitor, GI254023X, was described before.[21]

CXCL16 cleavage assays

TALDCs were grown in complete medium in six-well dishes for 24 h. The cells were washed with phosphate-buffered saline and 1 ml of fetal calf serum-free medium, with or without metalloproteinase inhibitor being added. After 10 min, cells were stimulated with IFN-γ (24 ng/ml) or left unstimulated for the indicated periods of time. The conditioned media were harvested and cleared by centrifugation. The presence of soluble CXCL16 in the conditioned media was quantified by a CXCL16-specific ELISA (PeproTech).

CXCL16-specific ELISA

The ELISA for human CXCL16 was conducted using Microlon 96-well plates (Greiner, Nurtingen, Germany) at room temperature, the reaction volume being 100 µl. The human CXCL16 ELISA Development kit from Peprotech was used according to the manufacturer's manual. All samples were tested in duplicates.

Western blot analysis

Cell extracts were prepared at the indicated time points as described previously.[22] Proteins were separated under reducing conditions by electrophoresis on 10 or 12% SDS–polyacrylamide gel for detection of ADAM10 or CXCL16, respectively. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, Munich, Germany). The blots were blocked for 30 min with 5% (w/v) non-fat dry milk dissolved in TTBS (1 × TTBS: 20 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween-20). Blots were probed overnight with a polyclonal CXCL16 antibody or anti-ADAM10 antibody diluted in TTBS containing 2% (w/v) non-fat dry milk. After incubation for 30 min with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), blots were developed with an enhanced chemiluminescence system (Amersham, Munich, Germany) and exposed to Hyperfilm enhanced chemiluminescence system (Amersham).

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated through a series of graded concentrations of ethanol, and washed in 10 mM phosphate-buffered 150 mM saline, pH 7.4. Antigen retrieval was performed by incubating the tissue sections for 20 min in 0.01 M sodium citrate buffer, pH 6, in a microwave oven (500 W). Tissue sections were treated with 3% H_2O_2 in methanol for 30 min at room temperature. After blocking sections with phosphate-buffered saline containing 10% horse serum and 1% bovine serum albumin for 1 h, they were incubated with an avidin/biotin blocking kit (Linaris, Wertheim, Germany) following the manufacturer's protocol. The serial sections were incubated overnight at 4 °C with primary antibodies as indicated. After washing the slides, the Universal Quick kit (Linaris) was used to stain the kidney sections. As a substrate, the AEC Substrate kit from BioGenex (San Ramon, CA, USA) was used to detect the immune complexes. Slides were counterstained with hematoxylin (Roth, Karlsruhe, Germany). The sections were inspected with a Zeiss microscope coupled to a 12-bit digital image camera.

Fluorescence microscopy

For immunofluorescence analysis, tissue sections were deparaffinized as described above and antigen retrieval was performed by incubating the tissue sections for 20 min in 0.01 M sodium citrate buffer, pH 6, in a microwave oven (500 W). After incubation with blocking buffer (0.1% Triton X-100/phosphate-buffered saline containing 1% bovine serum albumin and 10% horse serum) for 1 h, tissue sections were incubated with the first antibodies (diluted in 1% bovine serum albumin/10% horse serum/phosphate-buffered saline/0.1% Triton X-100) as indicated. Following washing, bound antibodies were detected using Alexa 488-conjugated goat anti-mouse (Molecular Probes, Karlsruhe, Germany) or goat anti-rabbit Cy3 (Molecular Probes) secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma, Deisenhofen, Germany) and slides were mounted in Fluoromount G (Southern Biotechnology, Birmingham, AL, USA). Evaluation was performed by fluorescence microscopy (Keyence, Neu-Isenburg, Germany) or slides were analyzed with an LSM 510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Chemotaxis assay

The chemotaxis of Jurkat T cells in response to recombinant CXCL16 or supernatants of siRNA-transfected primary TALDCs was measured as described before,[23] in which recombinant CXCL16 (100 ng/ml) or serum-free supernatants of siRNA-transfected TALDCs were added to the lower chamber in a volume of 600 µl. The plates were incubated for 2 h at 37 °C in 5% CO_2. Migrated cells were counted per visual field using inverted microscope at an original magnification of × 200 and the experiments were repeated twice in triplicates.[23]

Statistical analysis

The number of experiments, which were performed either in duplicate or triplicate, is reported in the legends to the figurers. Data are presented by means ± s.d. Statistical and significant differences were determined by analysis of variance with Bonferroni's multi-comparison test.

DISCLOSURE

All the authors declared no competing interests.

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REFERENCES

1. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. *Immunol Rev* 2003; **195**: 58–71.
2. Bazan JF, Bacon KB, Hardiman G *et al.* A new class of membrane-bound chemokine with a CX3C motif. *Nature* 1997; **385**: 640–644.
3. Matloubian M, David A, Engel S *et al.* A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol* 2000; **1**: 298–304.
4. Wu T, Xie C, Bhaskarabhatla M *et al.* Excreted urinary mediators in an animal model of experimental immune nephritis with potential pathogenic significance. *Arthritis Rheum* 2007; **56**: 949–959.
5. Garcia GE, Truong LD, Li P *et al.* Inhibition of CXCL16 attenuates inflammatory and progressive phases of anti-glomerular basement membrane antibody-associated glomerulonephritis. *Am J Pathol* 2007; **170**: 1485–1496.
6. Minami M, Kume N, Shimaoka T *et al.* Expression of SR-PSOX, a novel cell-surface scavenger receptor for phosphatidylserine and oxidized LDL in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1796–1800.
7. Heydtmann M, Lalor PF, Eksteen JA *et al.* CXC chemokine ligand 16 promotes integrin-mediated adhesion of liver-infiltrating lymphocytes to cholangiocytes and hepatocytes within the inflamed human liver. *J Immunol* 2005; **174**: 1055–1062.

8. Abel S, Hundhausen C, Mentlein R *et al.* The transmembrane CXC-chemokine ligand 16 is induced by IFN-gamma and TNF-alpha and shed by the activity of the disintegrin-like metalloproteinase ADAM10. *J Immunol* 2004; **172**: 6362–6372.
9. Gough PJ, Garton KJ, Wille PT *et al.* A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. *J Immunol* 2004; **172**: 3678–3685.
10. Segerer S, Schlondorff D. Role of chemokines for the localization of leukocyte subsets in the kidney. *Semin Nephrol* 2007; **27**: 260–274.
11. Geissmann F, Cameron TO, Sidobre S *et al.* Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol* 2005; **3**: e113.
12. Johnston B, Kim CH, Soler D *et al.* Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. *J Immunol* 2003; **171**: 2960–2969.
13. van der Woude FJ, Deckers JG, Mallat MJ *et al.* Tissue antigens in tubulointerstitial and vascular rejection. *Kidney Int Suppl* 1995; **52**: S11–S13.
14. Scholz F, Schulte A, Adamski F *et al.* Constitutive expression and regulated release of the transmembrane chemokine CXCL16 in human and murine skin. *J Invest Dermatol* 2007; **127**: 1444–1455.
15. Shimaoka T, Nakayama T, Kume N *et al.* Cutting edge: SR-PSOX/CXC chemokine ligand 16 mediates bacterial phagocytosis by APCs through its chemokine domain. *J Immunol* 2003; **171**: 1647–1651.
16. Tabata S, Kadowaki N, Kitawaki T *et al.* Distribution and kinetics of SR-PSOX/CXCL16 and CXCR6 expression on human dendritic cell subsets and CD4+ T cells. *J Leukoc Biol* 2005; **77**: 777–786.
17. Wu T, Xie C, Wang HW *et al.* Elevated urinary VCAM-1, P-selectin, soluble TNF receptor-1, and CXC chemokine ligand 16 in multiple murine lupus strains and human lupus nephritis. *J Immunol* 2007; **179**: 7166–7175.
18. Baer PC, Nockher WA, Haase W *et al.* Isolation of proximal and distal tubule cells from human kidney by immunomagnetic separation. Technical note. *Kidney Int* 1997; **52**: 1321–1331.
19. Baer PC, Bereiter-Hahn J, Schubert R *et al.* Differentiation status of human renal proximal and distal tubular epithelial cells *in vitro*: differential expression of characteristic markers. *Cells Tissues Organs* 2006; **184**: 16–22.
20. Elbashir SM, Harborth J, Lendeckel W *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–498.
21. Ludwig A, Hundhausen C, Lambert MH *et al.* Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell surface molecules. *Comb Chem High Throughput Screen* 2005; **8**: 161–171.
22. Wiechen K, Sers C, Agoulnik A *et al.* Down-regulation of caveolin-1, a candidate tumor suppressor gene, in sarcomas. *Am J Pathol* 2001; **158**: 833–839.
23. Klawitter S, Hofmann LP, Pfeilschifter J *et al.* Extracellular nucleotides induce migration of renal mesangial cells by upregulating sphingosine kinase-1 expression and activity. *Br J Pharmacol* 2007; **150**: 271–280.