IL-18 is expressed in the intercalated cell of human kidney

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We determined the cellular location of interleukin-18 (IL-18) and caspase-1 and the purinergic receptor P2X7, two proteins necessary for its activation and secretion. The mRNA and protein of IL-18 were detectable in normal human kidney by means of polymerase chain reaction (PCR), *in situ* hybridization, and Western blot. Immunohistochemistry located IL-18 to nephron segments containing calbinbin-D28k or aquaporin-2 that suggest location in the distal convoluted and the connecting tubule and to parts of the collecting duct. IL-18 was not detected in the thick ascending limb of Henle. Confocal microscopy showed that IL-18 was expressed in cells negative for calbindin-D28k and for aquaporin-2 but positive for the vacuolar H^+-ATPase. This demonstrates that the intercalated cells produce IL-18. These segments were also positive for caspase-1 and P2X7 that are essential for IL-18 secretion. Our results show that IL-18 is constitutively expressed by intercalated cells of the late distal convoluted tubule, the connecting tubule, and the collecting duct of the healthy human kidney. Since IL-18 is an early component of the inflammatory cytokine cascade, its location suggests that renal intercalated cells may contribute to immediate immune response of the kidney.

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Interleukin-18 (IL-18) can induce a wide variety of proinflammatory mediators that are relevant for the progression of various renal diseases. Some of these mediators are tumor necrosis factor-α, interleukin-1β, nitric oxide,[1] adhesion molecules,[2] chemokines,[3] and matrix metalloproteinases.[4] IL-18 shares some structural and functional proinflammatory properties with interleukin-1, but has the unique capacity to induce T-helper 1 or T-helper 2 polarization,[5] depending on the immunologic context. Recent findings suggest a role of IL-18 in certain renal diseases. Specifically, IL-18 function has been associated with lupus nephritis,[6] anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis,[7] acute kidney allograft rejection,[8] minimal-change nephrotic syndrome,[9] and also with acute tubular necrosis.[10] Although in all these cases increased expression of IL-18 in renal tissue or enhanced urinary excretion could be observed, a causal link between IL-18 expression and the progression of the diseases has not been demonstrated.[11]

Recently, Striz *et al.*[8] described IL-18 in cells of the distal tubule in transplant kidneys. The distal convoluted tubule (DCT) is composed of tubular cells responsible for the fine regulation of water and electrolyte balance. In addition, intercalated cells occur in the late part of this segment and are involved in the regulation of the acid–base balance. These intercalated cells are further present in more distal parts of the tubular system, for example, the connecting tubule (CNT) and the collecting duct (CD), and are separated from each other by the segment-specific cells.

A striking difference between IL-18 and most other proinflammatory cytokines lies in the fact that it is already constitutively expressed in a wide variety of different cell types such as peripheral blood monocytes,[3] microglia cells,[12] keratinocytes,[13] as well as in renal tubular cells.[8] Given its constitutive expression, it can be assumed that processing and release of preformed IL-18 from renal cells may participate at an early time point in the development of various renal diseases. Therefore, it is of interest to identify those renal cell types that synthesize and store IL-18 under normal conditions.

RESULTS

To detect IL-18 mRNA in normal human kidney tissue, we performed reverse transcriptase-polymerase chain reaction (PCR). We could amplify PCR products of the predicted size (598 bp) from cDNAs prepared from healthy parts of four individual tumor nephrectomies. In addition, caspase-1 and the purinergic receptor P2X7, two essential components of the IL-18 secretion pathway, were amplified from the same cDNAs (Figure 1a). The presence of IL-18 protein was proven by Western blot (Figure 1b). IL-18 protein was detected in lysates of the same four nephrectomy specimens used for PCR. In all cases, the antibody detected the 24 kDa form of IL-18, representing the biologically inactive pro-IL-18.

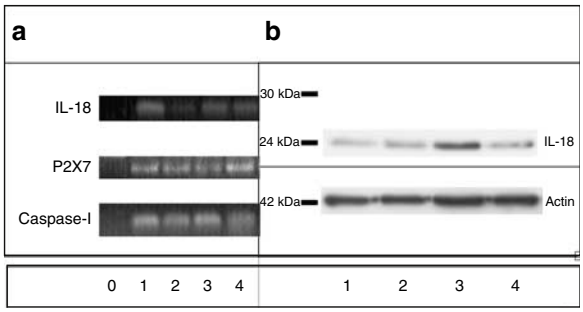

Figure 1 | IL-18 mRNA and protein expression in adult human kidney. Protein and RNA was extracted from homogenates of the nonaffected parts from four individual tumor nephrectomies (lanes 1–4) and analyzed for (**a**) IL-18 mRNA expression by reverse transcriptase-polymerase chain reaction and for (**b**) IL-18 protein content by Western blot analysis. In (**a**), lane 0 designates the control, where no enzyme was added to the reverse transcriptase reaction. Additionally, P2X7 and caspase-1 can be amplified from IL-18-positive cDNAs. (**b**) IL-18 protein is detected in all four human renal tissue samples.

Renal expression of IL-18 was further analyzed by immunohistochemistry. IL-18 was detected in distinct cells of different nephron segments (Figure 2). In these cells, IL-18 staining was located in the cytoplasm. In the renal cortex, IL-18 was detected in DCTs, CNTs, and CDs of the cortical labyrinth (identified by their morphological appearance; Figure 2a and b). In arcade-forming tubules, distinct IL-18-containing cells are observed (Figure 2b). These arcades represent parts of the CNT and the CD. IL-18 staining was not observed in any other structures of the renal cortex. Moreover, a subset of cells from CD profiles of medullary rays (Figure 2c) and the outer medulla exhibited IL-18 staining (Figure 2d). Detection of IL-18 expression extended toward the inner medulla (Figure 2e and f). However, only few tubular profiles were still IL-18-positive in the most distal part of the inner medulla (Figure 2g).

In accordance with the immunohistochemical findings, IL-18 mRNA could be detected by *in situ* hybridization in individual cells of distinct tubular profiles (Figure 2h and i).

To identify the exact cellular sites of IL-18 expression along the nephron, we performed double staining experiments with antibodies directed against IL-18 together with an antibody specific for calbindin-D28k as a marker for the DCT, the CNT and the proximal parts of the CD. In addition, an antibody against aquaporin-2 served as a marker for segment-specific cells of the CD. Third, a Tamm–Horsfall protein-specific antibody was used as a marker for the thick ascending limb of the loop of Henle in double-staining

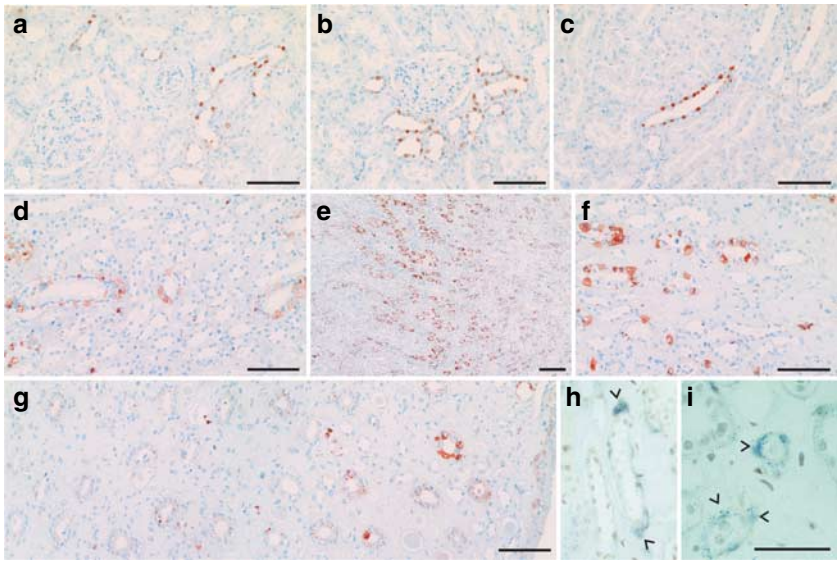

Figure 2 | Intrarenal distribution of IL-18 in the adult human kidney as determined by immunohistochemistry. (**a**, **b**) Kidney cortex, intense intracellular IL-18 staining (red signal) is confined to scattered cells of DCTs already passing into CNTs thereby forming arcades (in **a** on the right side). No signal is observed in glomeruli and proximal tubules. (**b**) Numerous DCTs and CNTs, and initial parts of CDs in the vicinity of a glomerulus exhibit focal staining for IL-18. (**c**) Medullary ray, expression of IL-18 is confined to cells of a CD profile. (**d**) In the outer medulla CD profiles show IL-18-positive signals. (**e**, **f**) At the transition from outer medulla to inner medulla numerous CD profiles are IL-18-positive (overview in **e**, and detailed view in **f**). (**g**) Inner medulla, including the papillary surface epithelium is shown. Some CD profiles exhibit immunostaining for IL-18. (**h**, **i**) Non-radioactive *in situ* hybridization for IL-18 mRNA. Isolated cells of CD in outer (**h**) medulla and (**i**) cortex express IL-18 mRNA (blue signal, arrows). Scale bars represent 100 μm.

experiments.[14] As shown in Figure 3, IL-18 staining (Figure 3a,c,e) was exclusively present in segments staining for calbindin-D28k (Figure 3b) and aquaporin-2 (Figure 3d). IL-18 was not observed in Tamm–Horsfall protein-positive nephron segments (Figure 3f).

A semiquantitative analysis of at least 1235 tubular cross sections per cellular marker was performed for renal cortex and outer stripe. IL-18 was nearly exclusively expressed in nephron segments that also expressed calbindin-D28k. Analysis of 96 slides from six individual specimens revealed that $96 \pm 4\%$ (mean $\pm$ s.d.) of the IL-18-positive tubules also expressed calbindin-D28k, whereas $76 \pm 22\%$ of the aquaporin-2-positive tubular profiles contained IL-18-positive cells.

The observed staining pattern suggests that IL-18 is expressed in intercalated cells. This assumption was supported by analyzing individual nephron segments by confocal laser scanning microscopy. IL-18 staining was strictly confined to the cells that do not express calbindin-D28k (Figure 4a–c) or aquaporin-2 (Figure 4d–f). The apical staining pattern of aquaporin-2 was interrupted by IL-18-positive cells in these segments (Figure 4e and f). IL-18 staining appears to fill the whole cytoplasm, but no nuclear staining was observed by confocal microscopy (Figure 4g–i).

The localization of IL-18 in intercalated cells was furthermore confirmed by double staining with V-H$^+$ATPase (Figure 5), which is expressed only in the intercalated cells of these tubular segments. The expression of IL-18 was not confined to a specific subtype of these cells, since IL-18 staining was present in A-type and non-A–non-B-type intercalated cells (expressing V-H$^+$ATPase apically) as well as in B-type intercalated cells (expressing V-H$^+$ATPase basolaterally).

Two essential components of the IL-18 processing and secretion pathway are caspase-1 and the purinergic receptor P2X7. As shown in Figure 6a and b, caspase-1, which cleaves the premature 24 kDa IL-18 and generates the active 18 kDa form, was present in all tubular segments that express IL-18. Similarly, P2X7 was present in IL-18-positive nephron profiles (Figure 6c and d). However, the expression of these two molecules was not limited to the IL-18-positive cells.

To exclude that the IL-18-positive nephron segments are in some inflamed state, we performed double stainings using antibodies against the p65 unit of nuclear factor-κB (NF-κB) and IL-18. Although, the segments expressing IL-18 (Figure 5e) intensely stained for NF-κB (Figure 6f), no nuclear localization of NF-κB was detected (Figure 6g). This indicates that NF-κB is not activated in these cells.

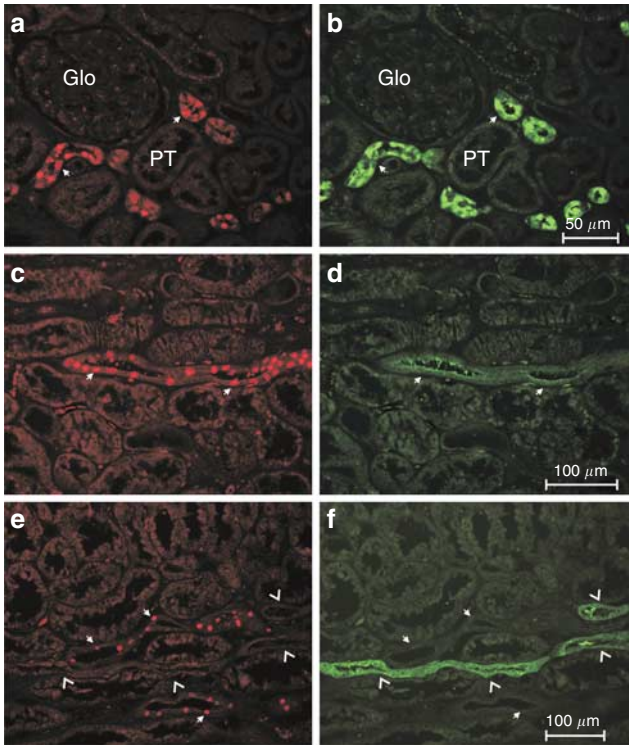

Figure 3 | Immunofluorescent detection of IL-18 in the adult human kidney together with segment-specific tubular markers. Double labelings for (**a**, **c**, **e**, red fluorescence) IL-18, and for (**b**) calbindin-D28k, (**d**) aquaporin-2, and (**f**) Tamm–Horsfall protein, all in green fluorescence. (**a**, **b**) Kidney cortex—IL-18 (**a**) is expressed in the same tubular profiles expressing calbindin-D28k (**b**), which are DCTs, CNTs, and initial parts of the cortical CD, examples are marked by arrows. Proximal tubules (PT) and glomeruli (Glo) are devoid of any signal. (**c**, **d**) Medullary ray—IL-18 (**c**) is exclusively expressed in a CD profile (arrows), characterized by aquaporin-2 immunoreactivity (**d**), (**e**, **f**) Medullary ray—IL-18 is expressed in CDs (**e**) (solid arrows), but not expressed in thick ascending limbs of the loop of Henle which are Tamm–Horsfall protein-positive (**f**) (open arrows). In (**e**) and (**f**) no overlap of both markers is observed, when the respective tubules are compared.

DISCUSSION

IL-18 is a key mediator of innate and acquired immunity. Recent reports addressing the role of IL-18 in kidney diseases indicate an involvement of this cytokine in acute tubular necrosis and renal vasculitis.[7,15] However, its significance for transplant rejection is at present not fully established.[8,16,17] IL-18 is constitutively expressed and intracellularly stored. Only processing and release require an appropriate stimulus. Therefore, we investigated what types of renal cells contain IL-18 in the healthy human kidney. IL-18 is produced as a 24 kDa precursor and is cleaved by caspase-1 to generate a biologically active, mature 18 kDa moiety.[18] In healthy human kidneys, only the 24 kDa premature form of IL-18 was detectable, although the antibody also detects the processed form of this cytokine. Occurrence of mature IL-18 is likely to be associated with inflammatory stimuli such as TLR ligands,[19,20] especially since a key mediator in the activation of epithelial cells in the context of innate immune responses, TLR-4, is present in distal parts of the nephron.[21] In the normal human kidney, IL-18 was present in cells of the DCT, the CNT, and the cortical CD as well as in portions of the medullary CD. In contrast, we did not detect significant IL-18 staining in glomeruli, proximal tubular cells, or endothelial cells. The spatial distribution of IL-18 expression along the tubular system could be defined by the

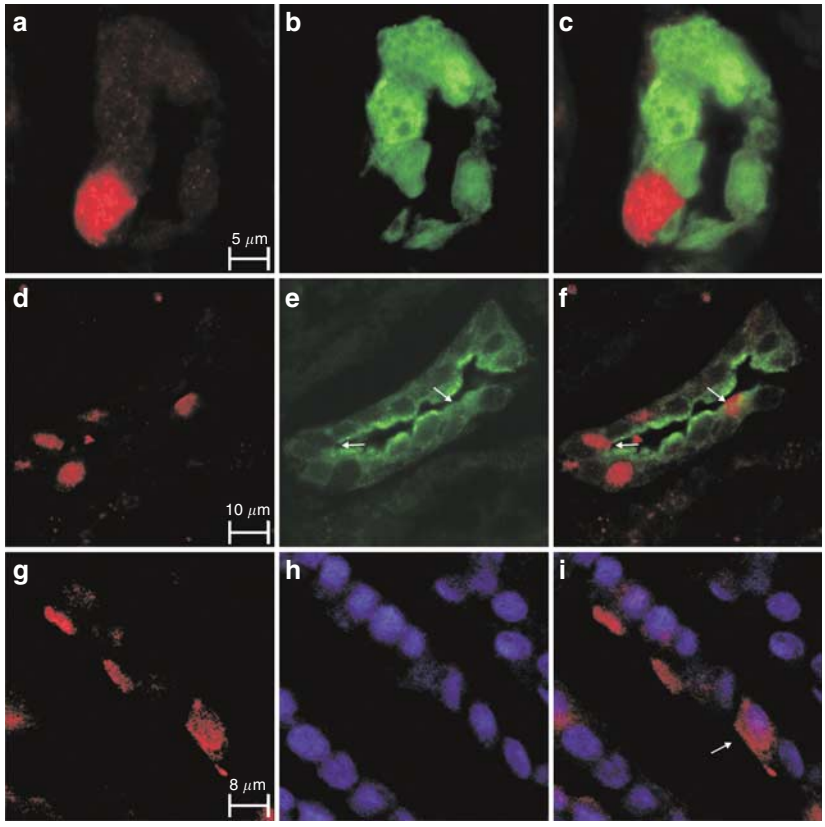

Figure 4 | Immunohistochemical expression of IL-18 in the intercalated cells of the adult human kidney, detailed view using confocal laser scanning microscopy. Double staining of IL-18 (red fluorescence, **a**, **c**, **d**, **f**, **g**, **i**) and either calbindin-D28k (green fluorescence, **b**, **c**) or aquaporin-2 (green fluorescent, **e**, **f**) or nuclei (blue TO-PRO-3 fluorescence, **h**, **i**). (**a**–**c**) A CNT profile. A cytoplasmic expression of IL-18 (**a**) is observed in a cell devoid of calbindin-D28k staining, whereas all calbindin-D28k-positive cells (**b**) do not express IL-18. IL-18 and calbindin-D28k staining are merged in (**c**). (**d**–**f**) A cortical CD profile with cytoplasmic expression of IL-18 (**d**) in isolated cells. These cells are not segment-specific cells stained by aquaporin-2 (**e**), since the latter apical expression is interrupted (arrows) by an IL-18-positive, aquaporin-negative cell. (**f**) Merged view of IL-18 and aquaporin-2 staining. (**g**, **i**) In individual cells of a CD, a cytoplasmic IL-18 staining is observed, which is not detected in the nuclei (**h**) of the cells. Since the colors are clearly separated (arrow) a nuclear expression can be excluded.

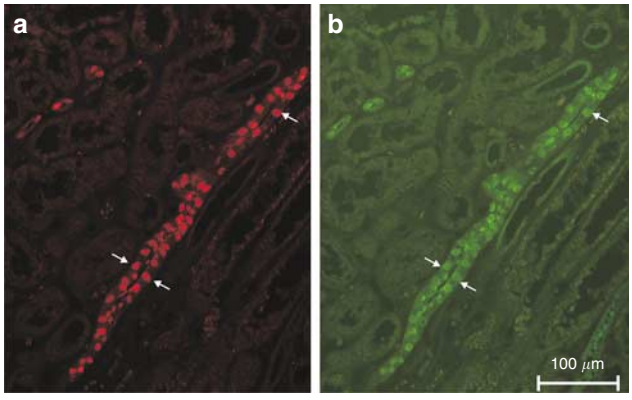

Figure 5 | Double immunofluorescent staining of IL-18 and V-H$^+$ATPase. A CD in a medullary ray is shown. (**a**, red) IL-18 is detected in cells that simultaneously express (**b**, green) V-H$^+$ATPase. The overlap of both signals (examples are marked by arrows) identifies these cells as intercalated cells.

concomitant expression of IL-18 and calbindin-D28k in the same tubular segments and the presence of IL-18 staining in most aquaporin-2-positive segments. These data indicate that IL-18 is expressed in a part of the tubular system that starts in the DCT and ends in the medullary CD.[14] These findings are in accordance with the morphological identification of the IL-18-positive segments. Since IL-18 staining is limited to distinct cells of a tubular profile, which are, on the one hand, negative for calbindin-D28k and aquaporin-2 and, on the other, positive for V-H$^+$ATPase, IL-18 expression can be clearly assigned to the intercalated cells that are the acid–base balance[22] regulating cell type of the nephron. Thus, these cells appear to be a major source of IL-18 in the kidney of healthy human subjects.

Expression of IL-18 in human kidney tissue and/or appearance in urine has been described previously in kidney allograft rejection,[8] renal anti-neutrophil cytoplasmic auto-antibody-associated vasculitis,[7] and in acute tubular necrosis.[10] In addition, increased expression was found in animal models of lupus nephritis and acute renal failure.[6,23] In renal tissue of healthy mice, a weak expression of IL-18 was detectable by Western blot and a cytoplasmic localization was described in tubular cells. In accordance with our findings, Striz *et al.*[8] described IL-18 expression in the distal tubules in protocol allograft biopsies of healthy renal transplant patients; however, cell types and exact localization were not

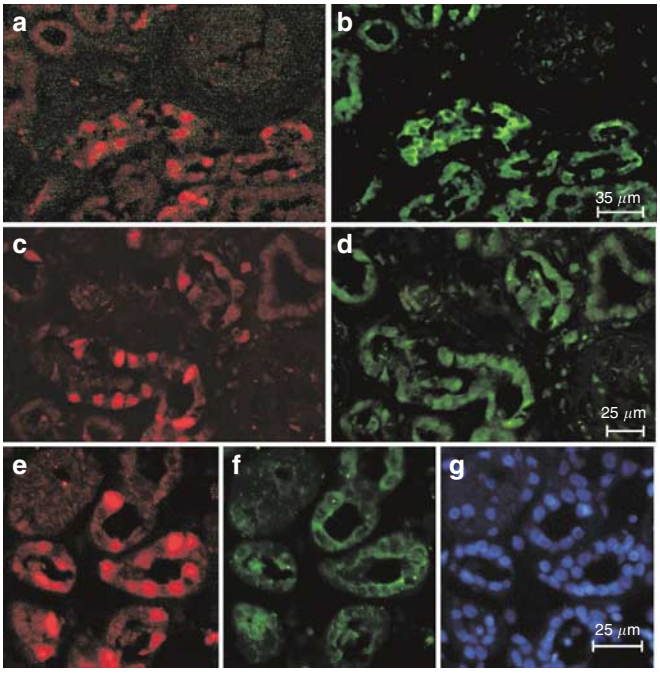

Figure 6 | Caspase-1 and P2X7 staining is observed in IL-18-positive nephron segments. Double staining with (**a** and **c**, red fluorescence) IL-18 and (**b**) caspase-1, or (**d**) P2X7 (both green fluorescence) exhibits that (**b**) caspase-1 and (**d**) P2X7 are expressed in all IL-18-positive tubular segments. However, both proteins are not restricted to IL-18-expressing cells. Double labeling with (**e**, red fluorescence) IL-18 and (**f**, green fluorescence) NF-κB demonstrates strong NF-κB staining in IL-18-expressing segments, but no nuclear localization of active NF-κB. (**g**) Nuclei are visualized by 4′,6′-diamidino-2-phenylindole staining.

further specified. In kidney transplants of patients undergoing an acute rejection also infiltrating leukocytes, proximal tubular cells, glomerular mesangial cells, and podocytes became positive for IL-18. Interestingly, in anti-neutrophil cytoplasmic autoantibody-associated systemic vasculitis podocytes are strongly positive for the IL-18 protein.[7] The potential of various renal cells to produce IL-18 in response to an external stimulus may account for the findings of Melnikov *et al.*,[23] who proposed proximal tubules as a renal source of IL-18 in mice. They reported that freshly isolated proximal tubules cultured under hypoxic or normoxic conditions produced IL-18. Provided that constitutive expression of IL-18 in kidney is similar between mouse and human, this finding may be attributed to an activation of the proximal tubules during the isolation procedure. While proximal[24,25] and distal[26] tubular cells as well as mesangial cells[27] have been shown to participate in immune reactions, there are only few data about CD cells, especially intercalated cells, and their role in an inflammatory response.

In an experimental mouse model of ascending urinary tract infection, an uropathogenic *Escherichia coli* strain bound preferentially to intercalated cells of the CD.[28] Furthermore, cultured monolayers derived from microdissected CDs responded with a TLR-4-dependent increase of proinflammatory mediators to a contact with several strains of *E. coli*. Among these mediators have been the chemokines monocyte chemoattractant protein-1 (MCP-1) and the mouse homolog of IL-8, macrophage inflammatory protein 2 (MIP-2), as well as the proinflammatory cytokines, TNF-α and IL-1β. Since IL-18 has been shown to induce IL-8 not only in cells of the immune system[29] but also in epithelial cells,[30] intercalated cells of the distal tubule and the CD may release IL-18 in response to bacteria and induce IL-8 production. IL-8 secreted from renal epithelial cells may recruit neutrophils into the renal tissue, thereby taking part in the immune defense against infectious organisms. This view is supported by our finding that two crucial components for the activation and release of IL-18, namely caspase-1 and P2X7,[31] are present in cells of these nephron segments. A major downstream signal of inflammatory signaling cascades is activation of NF-κB. In accordance with the reported role of CD cells in the defense against bacterial infection from the urinary space, we observed a high expression of NF-κB in tubular segments positive for IL-18. In the IL-18-expressing intercalated cells of normal human kidneys, however, we did not observe a nuclear localization of p65 NF-κB, which otherwise would indicate an activation of this signaling cascade. Therefore, we do not assume an inflammatory activation state of the IL-18-positive cells. Since this cell type contains large amounts of IL-18, intercalated cells may be involved in host defense against urinary tract infections.

Altogether, in this study, we demonstrated that intercalated cells in normal human kidney express IL-18. Moreover, these cells contain three major components required for the release of the active proinflammatory cytokine IL-18, namely pro-IL-18, P2X7, and caspase-1. Therefore, these cells might be capable to participate actively in renal immune reactions.

MATERIALS AND METHODS

Material

Specimens were taken from the healthy parts of six different tumor nephrectomies (obtained from two female and four male patients with ages from 34 to 66 years), which were originally submitted for diagnostic purposes and studied in accordance with national and local ethical principles.

Immunohistochemistry

Immunohistochemistry on paraffin-embedded tissue was essentially carried out as described previously.[32] Sections (3–4-μm thick) were deparaffinized and washed in distilled water, and finally equilibrated in 10 mM sodium citrate, pH 6.0, for 10 min. Next, slides were heated in a microwave oven at 500 W for 10 min and again for 10 min at 250 W. Thereafter, slides were allowed to cool to room temperature, rinsed in phosphate buffered saline (PBS) three times and incubated in blocking solution containing 100% fetal calf serum for 30 min in a humid chamber. Three different primary anti-human IL-18 antibodies were used: a monoclonal mouse antibody (ImmunoTools, Friesoythe, Germany) and two polyclonal rabbit antibodies (R&D Systems, Minneapolis, MN, USA and PeproTec, Frankfurt, Germany). In all stainings presented, the ImmunoTools

antibody has been used. The other two antibodies gave qualitatively the same results. The antibodies were applied for 1 h at 37°C and then kept overnight at 4°C, either alone (IL-18, 1:300) or in combination with rabbit antibodies against calbindin-D28k (Sigma, St Louis, MO, USA, 1:1000), aquaporin-2 (Sigma, 1:500), caspase-1 (Santa Cruz sc-622, Heidelberg, Germany, 1:120), P2X7 (Chemicon, Temecula, CA, USA, 1:50), V-H$^+$ATPase B1/2 (Santa Cruz sc-20943, 1:500), or Tamm–Horsfall protein (Biotrend, Köln, Germany, 1:500). The next day slides were rinsed three times in PBS and then incubated with a Cy3-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany, 1:500) alone or in combination with a FITC-labeled goat anti-rabbit antibody (Sigma, 1:20). To visualize the nuclei, sections were stained either with 2 μM TO-PRO-3 (Invitrogen, Karlsruhe, Germany) in PBS (for confocal microscopy) or with 1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma) in PBS (for conventional fluorescence) for 5 min before washing three times again with PBS.

NF-κB staining was performed using a rabbit antihuman antibody (Santa Cruz, sc-109, 1:50) Control incubations were performed with normal mouse or rabbit serum or blocking solution alone instead of the primary antibody. Photomicrographs were taken with a Leica RB fluorescence microscope. Confocal images were acquired using a Zeiss SM-510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Each channel was scanned independently to avoid crosstalk (Multi-Tracking). Horizontal (x, y) optical slices were obtained with a thickness of 1–2.5 μm. Colocalization experiments were studied in single x–y optical sections and merged using the Zeiss LSM software.

Peroxidase staining

After deparaffination and antigen retrieval by microwave treatment, the slides were treated with 3% hydrogen peroxide for 5 min at room temperature, rinsed with PBS, and blocked for 30 min with 20% human serum diluted in PBS. The IL-18 antibody (ImmunoTools, 1:300) was incubated for 1 h at 37°C and then kept overnight at 4°C.

After washing the slides with PBS, the biotinylated rabbit anti-mouse IgG (Dako, Hamburg, Germany, 1:250) was applied to it for 30 min at 37°C. They were again washed with PBS, and the slides were incubated for 30 min with ExtrAvidin-Peroxidase (Sigma, 1:100). The washing was repeated and then the peroxidase was detected by 3-amino-9-ethylcarbazole chromagen (Sigma, 0.8 mg/ml in 0.05 M acetate buffer, pH 5.0, 0.03% H_2O_2).

In situ hybridization

A 598-bp human IL-18 cDNA was amplified by reverse transcriptase-polymerase chain reaction from RNA of human kidney homogenates using primers obtained from the published sequences. The PCR fragments were cloned into a pCRII-TOPO vector (Invitrogen). Sense and antisense IL-18 cRNA probes were generated using a RNA digoxygenin labeling kit (GE Healthcare, München, Germany).

Sections (6 μm) of formalin-fixed, paraffin-embedded human kidney sections were dewaxed, rehydrated, proteinase K-treated (10 μg/ml), dehydrated, and hybridized overnight at 42°C using a hybridization mix containing 50 ng/section digoxigenin-labeled riboprobe, 50% deionized formamide, 4 × standard sodium citrate, 10 μg/ml heparin, 25 μg/ml yeast tRNA, 0.2% blocking reagent (Boehringer Mannheim, Roche, Grenzach-Wyhlen, Germany). Both antisense and sense digoxigenin-labeled human cRNAs were detected after RNase A treatment (10 μg/ml, 30 min) and stringency wash (0.1 × standard sodium citrate) with an alkaline phosphatase-coupled antidigoxigenin antibody (Dako) using NBT/BCIP as substrate. No staining could be detected in control hybridizations using equal amounts of IL-18 sense cRNA.

PCR

Nonaffected parts of tumor nephrectomies were snap-frozen in liquid nitrogen, ground, and homogenized in 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 100 mM 2-mercaptoethanol. RNA was prepared according to Chomczynski and Sacchi.[33]

IL-18, caspase-1, and P2X7 were amplified by reverse transcriptase-polymerase chain reaction using following primers: IL-18 forward primer, 5′-CAGACCTTCCAGATCGCTTC-3′; IL-18 reverse primer, 5′-CCCCCAATTCATCCTCTTTT-3′ (50°C, 30 cycles); caspase-1 forward primer, 5′-GTTTCTTGGAGACATCCC-3′; caspase-1 reverse primer, 5′-TAATGTCCTGGGAAGAGG-3′ (54°C, 35 cycles); P2X7 forward primer, 5′-CTGTCCCCAGGAAGTTGTGT-3′; P2X7 reverse primer, 5′-GCGAGTCTGGTCTTGGACTC-3′ (52°C, 30 cycles). PCR was performed using the indicated annealing temperatures.

Western blot

The unaffected parts of renal tumor nephrectomy specimen were snap-frozen in liquid nitrogen and stored at −80°C. Next, individual tissue samples were first ground in liquid nitrogen and then homogenized in sodium dodecyl sulfate sample buffer (62.5 mmol/l Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol) using a potter homogenizer. Protein content was determined by a BCA assay (Pierce, Rockford, IL, USA). After 10 min incubation at 95°C, 40 μg of total cellular protein from an individual tissue sample was loaded onto a 7.5% sodium dodecyl sulfate gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotter and a transfer buffer containing 48 mM Tris, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol. The blots were blocked with 5% fat-free dry milk in Tris-buffered saline, 0.1% Triton X-100, 0.05% Tween-20 (TTBS) for 2 h and incubated with the mouse anti-human IL-18 antibody (ImmunoTools antibody, 3.5 μg/ml) overnight at 4°C. After washing with TTBS, the blots were incubated with a rabbit anti-mouse antibody coupled with horseradish peroxidase (Amersham, Piscataway, NJ, USA) diluted 1:5000 in Tris-buffered saline. After washing the blots, the enzyme was visualized using an ECL Kit (Amersham).

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