Renal medullary osmolytes NaCl and urea differentially modulate human tubular cell cytokine expression and monocyte recruitment

Jessica Schmitz#1, Nicolas Brauns#2, Anne M. Hüsing2, Martina Flechsig2, Thorsten Glomb3, Jan Hinrich Bräsen1, Hermann Haller2 and Sibylle von Vietinghoff2,4

1 Nephropathology Unit, Institute for Pathology, Hannover Medical School, Hannover, Germany
2 Department of Internal Medicine, Division of Nephrology and Hypertension, Hannover Medical School, Hannover, Germany
3 Core Facility Transcriptomics, Hannover Medical School, Hannover, Germany
4 Nephrology Section, First Medical Clinic, University Clinic and Rheinische Friedrich-Wilhelms Universität Bonn, Bonn, Germany

Renal immune cells serve as sentinels against ascending bacteria but also promote detrimental inflammation. The kidney medulla is characterized by extreme electrolyte concentrations. We here address how its main osmolytes, NaCl and urea, regulate tubular cell cytokine expression and monocyte chemotaxis. In the healthy human kidney, more monocytes were detected in medulla than cortex. The monocyte gradient was attenuated in patients with medullary NaCl depletion by loop diuretic therapy and in the nephrotic syndrome. Renal tubular epithelial cell gene expression responded similarly to NaCl and tonicity control mannitol, but not urea. NaCl significantly upregulated chemotactic cytokines, most markedly CCL26, CCL2, and CSF1. This induction was inhibited by the ROS scavenger n-acetylcysteine. In contrast, urea, the main medullary osmolyte in catabolism, dampened tubular epithelial CCL26 and CSF1 expression. Renal medullary chemokine and monocyte marker expression decreased in catabolic mice. NaCl-, but not urea-stimulated tubular epithelium or CCL2 and CCL26, promoted human classical monocyte migration. CCL26 improved bactericidal function. In the human kidney medulla, monocyte densities correlated with tubular CCL26 protein abundance. In summary, medullary-range NaCl, but not urea, promotes tubular cytokine expression and monocyte recruitment. This may contribute to the pyelonephritis vulnerability in catabolism but can possibly be harnessed against pathologic inflammation.

Keywords: chemokine · monocyte · NaCl · renal tubular epithelium · urea

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Correspondence: Dr. Sibylle von Vietinghoff
e-mail: Sibylle.von_Vietinghoff@ukbonn.de

© 2022 The Authors. European Journal of Immunology published by Wiley-VCH GmbH.
www.eji-journal.eu
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
Introduction

To achieve up to 1200 mOsm in the urine, the human kidney establishes a marked osmotic gradient [1]. It is adapted to volume status and rises with the need for water conservation. The main medullary osmotic agents are sodium chloride (NaCl) and urea. Urea replaces NaCl as the main osmolyte in catabolism [2]. This includes the somewhat unexpected recent observation that a high salt diet induces a catabolic state that enhances medullary urea, rather than NaCl concentrations [2, 3]. Renal medullary NaCl is depleted by acute and chronic loop diuretic use [4–6] or in the nephrotic syndrome [7, 8].

The kidney medulla contains resident myeloid cells, namely monocytes and macrophages. Myeloid cells are central for the renal medullary immune response [9, 10]. Monocytes and macrophages recruit neutrophilic granulocytes, the body’s main line of immediate antibacterial response, into the urinary tract [11, 12]. On the other hand, renal macrophage densities predict the adverse outcome of inflammatory kidney conditions including autoimmune vasculitides and loss of transplant function [13].

Human gene expression and murine lineage tracking data suggest that renal myeloid cells are of embryonic and monocytic origin [14–17]. Marker analysis and bioinformatics estimates propose preferential localization of CD14+/monocytic cells to the medulla [11, 14]. Indeed, elevated NaCl concentrations enhance renal tubular epithelial cell secretion of the monocyte chemotactic cytokine CCL2 [11, 18]. How NaCl regulates other tubular cell cytokines and the underlying mechanisms has not been reported. Also, how the other main medullary osmolyte, urea, affects the tubular phenotype regarding chemokine expression has not been detailed.

We found that monocyte enrichment in the human kidney medulla varied with conditions that modulate local NaCl concentration. Therefore, we addressed mechanisms by which renal medullary-range NaCl and urea can affect monocyte migration. Medullary-range hypertonic NaCl, but not urea, dose- and time-dependently induced monocyte chemoattractant expression and recruitment by tubular epithelium. Our findings identify the human cytokine CCL26 as a novel cooperative monocyte chemoattractant that is induced by NaCl, but suppressed by medullary-range urea.

Results

Monocyte enrichment in the human kidney medulla correlates with the concentration gradient

Renal innate immune cells centrally modulate antibacterial host response as well as detrimental inflammation. We sought to morphologically define monocyte and macrophage distribution in the human kidney. Their abundance was assessed in histologically normal human kidney tissue (Fig. 1A). We used digitally assisted pathology of whole slide scans as previously described and detailed in methods to quantify infiltrates [18, 19]. CD14+ monocytes were significantly more abundant in medulla than cortex (Fig. 1A-C). This confirms measurements and bioinformatics estimates in other cohorts [11, 14]. The densities of CD68+ macrophages did not differ significantly between the compartments (Fig. 1B and C). Very few neutrophils were found (Supporting information Fig. S1).

We previously demonstrated induction of tubular monocyte chemotactic chemokine CCL2 expression by NaCl [18]. To determine whether renal monocyte distribution associates with the medullary salt gradient, we identified patient groups with clinical conditions known to alter the salt gradient that were sufficiently frequently biopsied and assessed monocyte densities in their kidney compartments.

First, loop diuretic therapy depletes the medullary salt gradient [4–6]. Its effect on monocyte distribution to cortex and medulla was studied in early surveillance biopsies of renal allografts. Clinical characteristics are given in Supporting information Table S1. Immunosuppressive regimens were very similar. There was no significant difference in rejection frequency or other characteristics defined by the Banff classification. Absolute monocyte densities were similar and higher in medulla than cortex in both subgroups (Fig. 1D, Supporting information Fig. S2). In addition, relative medullary CD14+ monocyte accumulation was significantly more marked in grafts without diuretic therapy (Fig. 1E). This occurred despite the fact that loop diuretic recipients’ grafts were more often cadaveric and had lower excretory function. The finding is consistent with a role of the NaCl gradient in their accumulation.

Second, the renal salt gradient is depleted in the nephrotic syndrome [7, 8]. We, therefore, studied renal monocyte and macrophage distribution in minimal change disease. Native kidney biopsies with an exclusive diagnosis of minimal change disease in patients without concomitant immunosuppression were assessed (Fig. 1F and G, clinical characteristics in Supporting information Table S2). Already in patients without diuretic therapy at the time of biopsy, absolute medullary CD14+ monocyte density and relative accumulation compared to the cortex were significantly less than in healthy controls (Dunn’s after Kruskal–Wallis test, Fig. 1C and G). In the subgroup of minimal change disease patients who received a loop diuretic at the time of biopsy, the medullocortical monocyte gradient was no longer significant (Fig. 1G).

Taken together, medullary CD14+ monocyte enrichment was observed in human kidneys. It was dampened and even abolished in conditions known for a reduced medullary salt gradient.

Equiosmolar NaCl and urea differentially regulate renal tubular epithelial gene expression

Induction of tubular chemotactic chemokine CCL2 expression by NaCl [18] leads to the hypothesis that the association of medullary monocyte accumulation with the concentration gradient in vivo was functionally mediated by tubular cell chemokine production. To systematically investigate tubular cell response to
Figure 1. Monocyte distribution in the human renal cortex and medulla and its modulation by loop diuretic therapy. (A–C) CD68^+ macrophages and CD14^+ monocytes were evaluated in histologically normal human kidney sections. Their abundance as % of all tissue was evaluated in cortex and medulla separately (A, examples; B, statistical evaluation of n = 8 patients each evaluated once, paired t-tests, bars indicate 100 μm). (C) The medullary-cortical gradients of CD68^+ macrophage and CD14^+ monocyte densities were compared in each individual kidney (n = 8 patients, each evaluated once, paired t-test). (D,E) CD14^+ monocyte medullary-cortical density gradients in human kidney allografts were compared in early surveillance biopsies of patients with and without loop diuretic therapy (D, examples, bars indicate 100 μm; E n = 12 and 15 patients per group, each evaluated once, t-test with Welch’s correction). (F,G) CD14^+ monocyte and CD68^+ macrophage medullary-cortical density gradients in human primary minimal change disease specimens were compared in patients without (n = 7 patients, each evaluated once) and with loop diuretic therapy at time of biopsy (n = 4 patients, each evaluated once) (F, paired t-tests; G, examples, bars indicate 100 μm). Data are expressed as mean ± SEM.
Figure 2. The effect of NaCl and urea on tubular epithelial cell gene expression. (A–D) Human renal tubular epithelial cells (HK2) were exposed to additional 120 mM NaCl, isosmolar urea (240 mM), or mannitol (240 mM) as tonicity control. Gene expression was assessed by gene array analysis after 24 h (n = 1 per condition in one experiment). (A) Venn’s diagrams of genes regulated twofold or more in parallel are shown for all, up- and downregulated genes of the three hyperosmolar conditions compared to normal media. The proportion of genes regulated by NaCl and urea in the up- and downregulated groups was compared by Fisher’s exact test. (B, C) Principal component analysis of all regulated genes (B) and with the variance threshold = 0.4 (C). (D) Heat map analysis, variance threshold = 0.4.

The increase in ambient NaCl and urea, we conducted gene expression studies.

HK2 human renal tubular epithelial cell gene expression was assessed after 24 h of culture with either an additional 120 mM NaCl or equiosmolar 240 mM urea, and compared to standard media and 240 mM mannitol as a tonicity control. Genes regulated more than twofold in relation to normal media were compared for the three hyperosmolar conditions (Fig. 2A). There was a major overlap between the hypertonic stimuli mannitol and NaCl, and markedly less with equiosmolar urea. This was also evident in principal component analyses (Fig. 2B and C). Similarly, heatmap analysis shows that a large proportion of the genes upregulated by NaCl was also upregulated by increased tonicity in the mannitol condition (Fig. 2D). The response to urea was more similar to the standard media control. It is of note that parallel regulation by NaCl and urea was significantly less common in the up- than in the downregulated fraction of genes (Fig. 2A). These data delineate a distinct tubular cell response to the main medullary osmolytes NaCl and urea.

NaCl, but not urea, induces tubular cell chemokine expression

To address tubular cell-derived recruitment cues in response to NaCl and urea, chemokine, cytokine, and IL expression was
Figure 3. Renal epithelial cell chemotactic cytokine expression response to elevated NaCl and urea. (A-E) Human renal tubular epithelial cells (HK2) were cultured with additional 120 mM NaCl, isoosmolar urea (240 mM), or mannitol as tonicity control. (A,B) Regulation of soluble immune mediators (chemokines, interleukins, and colony stimulating factors) was assessed by gene array analysis after 24 h (experimental conditions as in Fig. 2) and is shown relative to control (A, log values). All mediators regulated 1.5× or more are underlaid in gray. (B) Volcano plot analysis of iso- versus hypertonic osmolytes shows most upregulation in hypertonicity for CCL26 and CCL2, followed by CSF1. (C) Response of the three most-regulated mediators CCL26, CCL2, and CSF1 was assessed after 6, 24, and 48 h exposure to additional high (+120 mM) and low (+40 mM) NaCl concentrations and equiosmolar urea by qPCR (n = 3 in indep. exp., Dunnett’s after ANOVA at each time point). (D) HK2 were exposed to mannitol as tonicity control, NaCl, or urea of equal osmolarity. CCL26, CCL2, and CSF1 mRNA was determined by qPCR (n = 3 in indep. exp., Dunnett’s after ANOVA). (E) ROS-scavenger NAC (10 mM) was added to 24 h stimulation with the indicated osmolyte concentrations (120 mM NaCl, 240 mM urea, 60 mM NaCl + 120 mM urea) and chemokine mRNA assessed (n = 3 in indep. exp., Sidak’s test after ANOVA). Data are expressed as mean ± SEM.

analyzed in detail. Among all detected factors, 14 were up- and 9 downregulated 1.5× or more by hypertonic NaCl (Fig. 3A). Modulation by NaCl and mannitol was largely parallel, similar to what was observed for all genes (Fig. 2). CCL26, CCL2, and CSF1 were most differentially expressed in hypertonicity compared to controls (Fig. 3B). This was maximal after 24 h exposure (Fig. 3C). No such effect was observed if a lower amount of NaCl (+40 mM) or equimolar urea concentrations were added (Fig. 3C). Mannitol of equal tonicity induced these chemokines to a similar extent as NaCl, consistent with a tonicity-driven, substance-independent process (Fig. 3D). Indeed, scavenging of ROS with a known role in mediation of tonicity responses [20–22] using n-acetylcysteine (NAC) completely prevented NaCl-induced CCL26, CCL2, and CSF1 mRNA expression (Fig. 3E). These results introduce CCL26 as a NaCl-regulated chemokine and define a ROS-dependent tonicity response as the underlying mechanism.

The human kidney expresses CCL26 predominantly in the medulla

Indeed, CCL26 was expressed in the human kidney at both the protein and mRNA levels (Fig. 4A and B). To explore which of the NaCl-regulated mediators were present and similarly regulated in the human kidney, all chemotactic genes were upregulated at least 1.5× by NaCl (Fig. 3A), and were analyzed in healthy human medulla and compared to cortex (GSE3931) [23]. Among upregulated genes, CCL26, which was most enhanced by NaCl in tubular cells in vitro, also showed the strongest trend to increase in vivo (Fig. 4C). Among downregulated chemotactic factors, there was a similar medullary trend for IL32 (Supporting information Fig. S3A). We next stained for CCL26 in normal human kidney sections. It was mostly expressed by the proximal tubuli (Fig. 4D). Tubular CCL26 was significantly more abundant in medulla than
Figure 4. Renal chemotactic cytokine expression in cortex and medulla. (A,B) CCL26 protein (A) and mRNA (B) was assessed in normal tissues from tumor nephrectomies (n = 8 patients, each investigated once). (C) All chemotactic factors upregulated at least 1.5× by NaCl in tubular epithelium were analyzed in a human kidney outer cortex and inner medulla gene expression set (GSE3931). All genes reported by the array and their individual p-values are shown (n = 5 kidneys from 5 patients, each investigated once), log values relative to mean of cortex, whiskers represent total range, p-values of individual t-tests are shown if below 0.2. (D) CCL26 expression (red) in relation to proximal tubular markers CD10 and CD138, loop of Henle (LOH), and distal tubular marker EMA, marker of ascending LOH cytokeratin (CK)7, and collecting duct marker cytokeratin (CK) 34βE12 (green, blue: DAPI nuclear counterstain, examples of at least 10 HPF per marker, bars = 50 μm). (E,F) The proportion of CCL26+ tubuli in cortex and medulla of normal human kidney was assessed after immunostaining on digitalized images using an interactively set threshold based on diaminobenzidine optical density (E, examples of staining and tubular annotation, n = 8 patients, each tested once, Wilcoxon matched-pairs test, bar = 50 μm).
cortex (Fig. 4E and F), introducing CCL26 as a novel human kidney medullary chemokine.

**Suppression of tubular cytokine expression by urea**

The role of urea for human tubular cytokine expression was addressed next. Our gene expression screen suggested downregulation of several chemotactic factors by urea (Fig. 3A). The most regulated CCL26, CCL2, and CSF1 were studied further. The addition of 240 mM urea suppressed the effect of NaCl on CCL26 and CSF1, but not on CCL2 mRNA (Fig. 5A). To test whether this inhibition also applies to other CCL26 inducers, we employed IL-4 and IL-13 that promote CCL26 expression in other cell types [24–27]. IL13 expression was detectable in the normal human kidney medulla (GSE3931, data not shown). Both IL-4 and IL-13 markedly enhanced CCL26 expression in human renal tubular epithelium (Fig. 5B). There was a cooperative effect with elevated NaCl concentration (Supporting information Fig. S4). In contrast, medullary range urea significantly inhibited cytokine-induced tubular epithelial CCL26 (Fig. 5B). These data demonstrate the suppressive functions of the urea on the expression of distinct human tubular cell chemokines.

**Suppression of renal medullary cytokine expression in catabolism in vivo**

In vivo, urea accumulates in the renal medulla in catabolism, as investigated in detail in mice during a high salt diet [2]. As patients are rarely biopsied in this condition, we analyzed a renal gene expression dataset [21] of volume-depleted and severely catabolic mice after 72 h of water restriction (Fig. 6A). Their food intake was 19% of controls. Gene expression in cortex and inner stripe of outer medulla was compared in catabolic mice and controls. Consistent with published functional data [2, 3], urea, but not sodium transporter expression increased in this state (Supporting information Fig. S5). Overall, immune response pathways...
Figure 6. Regulation of monocyte chemotactic factors in the catabolic kidney medulla. (A–G) Gene expression in renal cortex and inner stripe of outer medulla was analyzed in catabolic (cat.) mice after 72 h of water deprivation and untreated controls (experimental setup in A, GSE81741). Macrophage marker CD68 and murine monocyte marker CD115 (Csfr1) (B), monocyte subtype markers and chemokine receptors CCR2 and CX3CR1 (C), M1 and M2 macrophase markers MHCII (H2Aa) and CD206 (Mrc2) (D), and cognate ligands (E) were assessed (n = 3 ctrl., n = 5 catabolic mice in one exp., Bonferroni after ANOVA). (F) Overview of murine chemokines and their receptors. Genes regulated in human tubular epithelium by NaCl and urea are marked in blue if detected in the murine kidney and red if upregulated in normal medulla compared to cortex and downregulated in the medulla in catabolism. Known interactions are marked by arrows. CCL26 is a pseudogene in mice. (G) Regulation of CCR3, which is among human CCL26 receptors, and its murine ligands CCL3, CCL4, and CCL5 mRNA expression in murine cortex and medulla at rest and in catabolism. (H,I) Total kidney gene expression after 4-week caloric restriction of 70% of normal food intake (H). Relative change and adjusted p-values of all genes analyzed in B–G are shown (I, chemokines in blue; receptors and surface markers in black; significant p-values are indicated above dotted line on y-axis. Ccl2, Ccl3, Ccl4, and Ccl5 were below detection limits, n = 4/group in one exp.). (J) Murine inner medullary collecting duct (IMCD) cells were exposed to the indicated additional NaCl and urea concentrations and Ccl2 and Ccl5 chemokine gene expression assessed after 24 h. Ccl3 and Ccl4 were below detection limit (n = 6 from 3 indep. exp., Dunnett’s after ANOVA). Data are expressed as mean ± SEM.

were prominently more expressed in normal than catabolic medulla (Supporting information Fig. S6A), which are consistent with an enhancement by NaCl and dampening by urea. NaCl-mediated upregulation would also imply that these genes should be expressed at higher levels in normal medulla than normal cortex. Indeed, genes upregulated in both comparisons, that is, normal medulla versus normal cortex and normal medulla versus catabolic medulla, contained similar functional groups (Supporting information Fig. S6B). A number of chemokines and receptors were detected (Supporting information Fig. S6C). Visualization of all common upregulated pathways in this double comparison, that is, in normal medulla compared to normal cortex and normal compared to catabolic medulla underlines a prominent position of immunoregulatory genes (Supporting information Fig. S6D).

Monocytic gene expression was analyzed based on the histologic results in the human kidney (Fig. 1, [11]). Macrophage marker CD68, murine monocyte marker CD115 (Csfr1), and non-classical monocyte/DC marker CX3CR1 were expressed in higher levels in the murine kidney medulla than in cortex in resting conditions, while classical monocyte marker CCR2 level was higher in the cortex (Fig. 6B and C). In catabolism, CD68 and CX3CR1 levels dropped significantly in the medulla. Also, expression of the
MHCI molecule H2Aa as the levels of M1 polarization marker dropped more than M2 marker CD206 in the medulla of catabolic mice (Fig. 6D), consistent with a regulation by NaCl and in parallel with our earlier results in the human kidney [18].

Soluble chemotactic factors produced by tubular epithelium were analyzed next (Fig. 6E). CCL26 is a pseudogene in mice [28]. Renal medullary CSF1 mRNA expression was downregulated in catabolism, but CCL2 and IL-1ß mRNA were not significantly affected when compared to control medulla. With the human NaCl-regulated chemokine system in view (Supporting information Fig. S3C), we investigated other human CCL26 receptors to explore for parallel responses in humans and mice that are observed for most types of inflammation [29] (Fig. 6F). Indeed, Ccr5 and its ligand Ccl3 were expressed significantly higher in resting medulla than cortex (Fig. 6G). This was abrogated in catabolism, consistent with a NaCl-dependent regulation.

To address whether this could be attributed to catabolism, a second independent dataset of more moderate catabolism was analyzed [30]. Here, 4-week caloric restriction decreased body weight to 76% and doubled serum urea (Fig. 6H). We analyzed regulation of chemokines and their receptors in the kidneys of these mice. All the chemokines and monocyte surface receptors that were decreased in the severely catabolic medulla (Fig. 6B-G) were also downregulated in the whole kidney of this model (Fig. 6I). Ccr2 and Ccr5 decreased significantly in both. Finally, we investigated the mechanistic role of urea in the murine system in inner medullar collecting duct cells. Indeed, both Ccl2 and Ccl5 were upregulated by NaCl, and the CCR5 ligand Ccl5 was significantly decreased by urea (Fig. 6J). In summary, depression of chemokine expression by urea in vitro reflected decreased chemokine and monocytic marker gene expression in the catabolic kidney medulla in vivo.

**NaCl-treated tubular epithelium and its cytokines promote monocyte migration and bacterial killing**

Effects of tubular cell stimulation by medullary-range NaCl on monocyte migration and function were studied in primary human cells in vitro.

To test for effects of tubular NaCl exposure on monocyte migration, we incubated tubular cells in medullary-range NaCl or equiosmolar urea for 24 h and placed monocytes in a transwell above. CD14+/CD16- monocytes migrated significantly more toward NaCl-stimulated than control or urea-stimulated epithelium (Fig. 7A and B, gating in Supporting information Fig. S7A), consistent with induction of chemotactic factors. To delineate the effects of the most-regulated CCL26 and CCL2, recombinant human cytokines were employed. While CCL2 function is well described in Ref. [31], less is known about CCL26. Among known human CCL26 receptors [28, 32, 33], expression of CCR1, CCR5, and CX3CR1, but not CCR2 or CCR3, was detected in the normal human medulla (Supporting information Fig. S3B; schematic depiction of putative receptor ligand interactions in Fig. S3C). Both CCL2 and CCL26 increased ERK phosphorylation in human monocytes (Fig. 7C). In addition, recombinant CCL2 and CCL2 cooperatively enhanced total and CD14+ monocyte migration (Fig. 7D and E). This is consistent with the functional collaboration of these chemokines to induce migration toward NaCl-stimulated tubular epithelium. To mimic the medullary situation, chemokine-induced monocyte chemotaxis was assessed in the presence of additional 120 mM NaCl. Also here, a combination of CCL2 and CCL26 significantly promoted their migration (Fig. 7F and G). CCL26 promoted bacterial killing by human blood monocytes (Fig. 7H and I). In summary, the data demonstrate a cooperative function of CCL2 and CCL26 on monocyte chemotaxis and promotion of bacterial killing.

**Tubular CCL26 associates with monocyte distribution in the human kidney**

To address a possible role of CCL26 for monocyte distribution in the human kidney, costainings of CD14+ monocytes and CD68+ macrophages with CCL26 were performed in human kidneys (Fig. 8A). Digitally assisted quantification shows that the proportion of CCL26 expressing tubuli in the renal medulla significantly correlated with medullary CD14+ monocyte densities in both, renal allografts (Fig. 8B) and native kidneys with minimal change disease (Fig. 8C). In contrast, there was no association with CD68+ macrophage densities (Fig. 8D and E) or macrophage polarization markers (Supporting information Fig. S8). Consistently, CCL26 did not affect M1 or M2 marker expression during macrophage differentiation from human monocytes in vitro (Supporting information Fig. S9). More monocytes were observed close to CCL26+ than CCL26− tubuli in the same kidney (Fig. 8F). CCL26 correlation with monocyte concentration is consistent with a functional role for their migration in the human kidney.

**Discussion**

Our data demonstrate that the chief renal osmolytes, NaCl and urea, exert differential effects on renal tubular epithelial cell chemokine production. They determine CCL26 as a novel NaCl-induced human cytokine with monocyte chemoattractive function.

The knowledge of the immunoregulatory roles of osmolarity and tonicity has rapidly advanced in recent years [34, 35]. Compared to other organs, the kidney reaches extreme osmolyte concentrations. In addition, it regulates the fluid and electrolyte balance of the whole body. Thus, the local milieu is the result of a complex interplay of systemic and local electrolyte systems. We addressed the roles of the main renal osmolytes, NaCl and urea [1, 2], for tubular cells as a major renal resident cell type producing chemotactic cytokines [11, 18] together with monocytes as major mobile effectors and regulators of renal antibacterial defense [12, 36], and also detrimental inflammation [13].

We found that in tubular epithelium, NaCl enhanced cytokine expression. These NaCl effects were largely substance
Figure 7. Monocyte migration, differentiation, and bacterial killing in response to NaCl-stimulated epithelium and induced chemokines. (A,B) Human renal tubular epithelial cells (HK2) were exposed to additional 120 mM NaCl, equiosmolar urea, or standard media for 24 h. Cells were washed and culture was continued in standard media for 3 h. During the last hour, human PBMCs were added in a transwell chamber. Monocyte migration was assessed by flow cytometry (experimental setup in A, B: statistical analysis of n = 4 from 2 indep. exp., Dunnett’s after ANOVA, gating in Supporting information Fig. S7A). (C) ERK phosphorylation was assessed in human peripheral blood CD11b+ mononuclear cells after 5 min exposure to recombinant CCL2 (10 ng/mL) or CCL26 (1000 ng/mL) (1 of 2 indep. exp., gating in Supporting information Fig. S7B). (D,E) Migration of monocytes in a transwell toward recombinant CCL2 (1 ng/mL) or CCL26 (500 ng/mL) was assessed by flow cytometry (experimental setup in D, E: statistical analysis of n = 4 from 2 separate exp., Dunnett’s after ANOVA). (F,G) Migration of monocytes in a transwell in media supplemented with 120 mM NaCl toward recombinant CCL2 (1 ng/mL) or CCL26 (500 ng/mL) was assessed after 1 h by flow cytometry (experimental setup in F, G: n = 4 from 2 separate exp., ratio paired t-tests). (H,I) Killing of Escherichia coli during 1 h coincubation with CCL26-prestimulated compared to control monocytes (experimental setup in H, I: n = 9 from 9 separate exp., paired t-tests). Data are expressed as mean ± SEM.

independent and similarly elicited by elevated tonicity, that is, by mannitol. This is similar to cytokine regulation in human intestinal and bronchial epithelial and PBMCs [37–39]. Chemokine induction required ROS, which agrees with mechanisms of hypertonic stimulation in other cell types and settings [20, 22, 40]. Our data introduce CCL26 as a novel monocyte attractant in the human kidney. It was strongly induced by NaCl in vitro and was prominent in the human renal medulla, adding renal tubular epithelium to the known CCL26 producers, bronchial epithelial cells [24, 25], endothelium [41], and fibroblasts [24, 26]. CCL26, which is a pseudogene in mice [28], was previously detected in human kidney transplants [42]. It was first described as a moderate eosinophil chemoattractant and later was found to also promote NK cell and monocyte migration [28, 43]. CCL26 functional effects appear to differ depending on the presence of other cytokines and whether recombinant or chemically synthesized protein is used [28, 32, 33]. Our results demonstrate a cooperative function with CCL2 in classical monocyte attraction. Recruited monocytic cells can replace myeloid cells of embryonic origin and develop similar phenotypes in the kidney, as demonstrated in murine models of cell fate mapping [16, 17]. Human renal myeloid cell fates are technically challenging to verify. Our data demonstrating that CCL26 modulates monocytic cell function toward more effective bacterial killing are consistent with a functional role of monocyte-derived cells in antibacterial response. Effects on other recruited myeloid cells, such as granulocytes, as major antibacterial effectors remain to be studied. CCL26’s murine functional homologues were similarly regulated.
Figure 8. Correlation of CCL26 and monocyte abundance in the human renal medulla. (A-E) CD14+ monocyte (B,C) and CD68+ macrophage (D,E) densities in renal medulla of renal transplant recipients (B,D) and patients with minimal change disease (C,E) are shown in relation to tubular CCL26 expression (A, examples of cortex in medulla with high and low CCL26 densities, cohorts as in Fig. 1 and Supporting information Tables S1,2). (F) The number of CD14+ monocytes within 10 μm of CCL26+ versus CCL26− tubules was compared (n = 15 tubuli from one patient evaluated once, Mann-Whitney test). Data are expressed as mean ± SEM.
in the kidney medulla in vivo and by NaCl and urea in murine renal epithelium in vitro, supporting a common regulatory pattern in these mammalian species. Consistently, monocyte, but not macrophage densities in the human renal medulla correlated with CCL26 protein and gradients were decreased in conditions with a loss of NaCl gradient.

Urea, the other main medullary osmolyte, either did not affect or even dampened tubular chemokine production, namely human CCL26 and murine Ccl5. These effects did not significantly depend on earlier defined mechanisms [44] (data not shown). Their underlying signaling remains to be determined. NaCl intake and hypernatremia cause catabolism in humans and mice [2, 3, 45] and increase renal medullary urea content [2, 3]. As no catabolic patient cohorts who underwent kidney biopsies were identified, we analyzed renal gene expression in two independent datasets of short- and long-term catabolic mice. Both show downregulation of chemokine and cognate receptor expression, most markedly with concomitant severe antidiuresis [21, 30]. This is consistent with tubular cell chemokine depression that was elicited by urea in both murine and human cells in vitro.

Systemic NaCl improves immune response to the most, but not all infections at sites other than the kidney [3, 46–49]. This applies especially to increased local NaCl concentrations, which were, however, mostly markedly lower than in our study. Our results are consistent with a local proinflammatory function of renal medullary-range NaCl by induction of tubular cell chemokine expression, extending earlier observations [11, 18], and possibly a previously described direct chemotactic effect [50]. By demonstrating that urea is dampening or inert, our data propose novel mechanisms about how catabolism decreases renal host defense. This project focused on chemokine regulation by urea and NaCl. Their role in the regulation of other immunoregulatory and inflammatory factors, including danger signaling and in different inflammatory renal diseases, remains to be detailed.

In summary, our results depict two different renal medullary scenarios depending on whether NaCl or urea is the main osmolyte. The energy intense state with tubular cell cytokine production and recruited myeloid cells in the presence of elevated NaCl may aid antibacterial host response. On the other hand, less cytokine production and a stable myeloid cell population in the presence of elevated urea concentrations may benefit an organism with limited energy supply [51]. It may also limit detrimental inflammation. This concept could provide a basis to additional management strategies of these patient populations.

**Materials and methods**

**Human renal tissue samples**

Normal renal tissue was identified in tumor nephrectomy specimens by a licensed renal pathologist (n = 8, 13% male, age 61.1 years, range: 39–75 years). Renal transplant surveillance biopsies were identified during a previous screening of all adult first renal transplant recipients at Hannover Medical School, conducted according to the declaration of Istanbul, in 2010 for patients who underwent surveillance biopsies. CD68 staining has been reported [19]. In the present study, all transplant biopsy samples with available cortex and medullary tissue were stained for CD14 (n = 27). Clinical chemistry values were assessed in the MHH clinical laboratories. Immunosuppressant and diuretic use and dose (furosemide or torasemide; torasemide dose was multiplied by four to express the biological equivalent) at first outpatient presentation were extracted from the clinical records. For the selection of minimal change disease samples, all kidney biopsies performed at MHH in adult patients with an exclusive diagnosis of minimal change disease from 2009 to 2019 were identified from the archives at the Institute for Pathology. Clinical data, immunosuppressants, and diuretics were extracted from the records. All patients (n = 11) with residual cortical and medullary tissue for staining who did not receive immunosuppressants at the time of biopsy were included in this study. Immunostaining and microscopy techniques are detailed in the online supplement.

**Human and murine cell culture, hyperosmolar stimulation, and bacterial killing**

HK2 human tubular cells and murine inner medullary collecting duct cells (IMCD, both: ATTC, Manassas, VA). A gene expression profile consistent with proximal tubular cells was ascertained in the gene array. Leukocytes and normal human serum were recovered from fresh blood or anonymized buffy coats obtained as a waste product from the blood donor service. Human PBMCs were enriched by density gradient centrifugation with Biocoll 1.077 (Biochrom, Berlin, Germany). Hyperosmolar stimulation and assessment of monocyte migration, macrophage differentiation, and bacterial killing are described in the online supplement.

**Quantification of mRNA and soluble protein**

RNA isolation, qPCR, and ELISA procedures were essentially as described [20] and are detailed in the online supplement together with the primer sequences.

**Microarray specification and gene expression dataset analyses**

A refined version of the whole human genome oligo microarray 4 × 44K (V2, ID 026652, Agilent Technologies) called “026652QM_RCUG_HomoSapiens” (ID084555) developed by the Transcriptomics Core was used as described in Ref. [52]. Data are available as GSE159238. For generation of heatmaps and
principal component analysis (PCA) plots, normalized microarray data were imported into OmicsExplorer (V3.3, Glocure).

Gene array data from normal human kidney (GSE3931) [23] and cortex and inner stripe of outer medulla of murine control and catabolic kidney (n = 3–5 female mice/group, GSE81741) [21] were obtained at NCBI. PANTHER overrepresentation test (V14.1, http://www.pantherdb.org) with Fisher’s exact test and Bonferroni correction and the Reactome analysis tool at reactome.org [53] were employed for the identification of functional gene groups. Calculation of -log10 adjusted p-values and log-fold changes of candidate genes of the long-term starvation RNA sequencing dataset compared to control mice [30] (n = 4 male mice/group) was performed with the public online application of this project.

Flow cytometry

Staining reagents are listed in the online supplement. Flow cytometry analysis was performed on a Becton–Dickinson FACSCanto (Franklin Lakes, NJ) according to the current guidelines [54]. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistics

Two-tailed student’s t-test with Welch’s correction, if variance was unequal, was used to compare two conditions using GraphPad-Prism Version 8.2.1 (Irvine, CA). If more than two conditions were compared, Bonferroni’s test of selected conditions or Dunnet’s test was applied after ANOVA or nonparametric test was employed as appropriate and indicated in the figure legends. Data are expressed as mean ± SEM, p-values <0.05 were considered significant and are indicated: *p < 0.05, **p < 0.01, ***p < 0.001.

Acknowledgments: We would like to thank all patients for making their samples available to research, T. Tuchel, E. Christians, M. Taleb-Naghsh, J. Jost, and the Laser Microscopy and Transcriptionomics Core facilities at Hannover Medical School for expert technical assistance. NB was supported by a Hannover Biomedical Research School stipend, JS and JHB by Jackstädt Stiftung and German Ministry for Education and Research (BMBF 13GW0399B), and SvV by grants from MHH Hilf2019 and MHH Transplant Centre and Deutsche Forschungsgemeinschaft (450775971 and EXC2151–390873048).

Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author contributions: JS, NB, HH, and SvV designed research; JS, NB, AMH, MF conducted experiments; JS, NB, TG, JHB, and SvV analyzed data; JS, NB, and SvV wrote the manuscript with help from all coauthors, all authors approved the manuscript.

Ethics approval statement: Leukocytes and normal human serum and renal biopsies were analyzed after local ethics board approval (MHH 807, 3516 and 10183).

Data availability statement: All datasets have been uploaded to a public database and will be made publicly available upon publication of the manuscript (GSE159238).

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202149723

References

1. Sands, J. M. and Layton, H. E. Advances in understanding the urine-concentrating mechanism. Am. Rev. Physiol. 2014: 76: 387–409.
2. Kitada, K., Daub, S., Zhang, Y., Klein, J. D., Nakano, D., Pedchenko, T., Lantier, L. et al. High salt intake reprioritizes osmolyte and energy metabolism for body fluid conservation. J Clin Invest. 2017: 127: 1944–1959.
3. Jobin, K., Stumpf, N. E., Schwesinger, C., Neubert, P., Rauh, M., Adamowski, M. et al. A high-salt diet compromises antibacterial neutrophil responses through hormonal perturbation. Sci Transl Med. 2020: 12: eaay3850.
4. Grist, J. T., Riemer, F., Hansen, E. S. S., Tougaard, R. S., McLean, M. A., Kaggie, J., Begh, N. et al. Visualization of sodium dynamics in the kidney by magnetic resonance imaging in a multi-site study. Kidney Int. 2020: 98: 1174–1178.
5. Sheen, M. R., Lim, J.-A., Lim, S. W., Jung, J.-Y., Han, K.-H., Jeon, U. S., Park, S.-H. et al., Interstitial tonicity controls TonEBP expression in the renal medulla. Kidney Int. 2009: 75: 518–525.
6. Sone, M., Albrecht, G. J., Dorge, A., Thurai, K. and Beck, F. X. Osmotic adaptation of renal medullary cells during transition from chronic diuresis to antidiuresis. Am J Physiol-Renal Physiol. 1993: 264: F722–F729.
7. Fernández-Llama, C. Mitochondria defect in experimental nephrotic syndrome: altered expression of aquaporins and thick ascending limb Na+ transporters. Kidney Int. 1998: 54: 170–179.
8. Matar, R. N. B., Malik, B., Wang, X. H., Martin, C. F., Eaton, D. C., Sands, J. M. and Klein, J. D. Protein abundance of urea transporters and aquaporin 2 change differently in nephrotic pair-fed vs. non-pair-fed rats. Am J Physiol. Renal Physiol. 2003: 302: F1545–F1553.
9. Ching, C., Schwartz, L., Spencer, J. D. and Becknell, B. Innate immunity and urinary tract infection. Pediatr Nephrol. 2020: 35: 1183–1192.
10. Abraham, S. N. and Xiao, Y. The nature of immune responses to urinary tract infections. Nat Rev Immunol. 2015: 15: 655–663.
11. Berry, M. R., Mathews, R. J., Ferdinand, J. R., Jing, C., Loudon, K. W., Wlodek, E., Dennison, T. W. et al., Renal sodium gradient orchestrates a dynamic antibacterial defense zone. Cell. 2017: 170: 860–874.
12. Schiwon, M., Weishit, C., Franken, L., Gutweiler, S., Dixel, A., Meyer-Schwesinger, C., Pohl, J.-M. et al., Crosstalk between sentinel and helper macrophages permits neutrophil migration into infected uroepithelium. Cell. 2014: 156: 456–468.

www.eji-journal.eu
Tissue immunology and leukocyte trafficking

Banwell, M. E.

Tang, P. M.-K., Nikolic-Paterson, D. J. and Lan, H.-Y. Macrophages: versatile players in renal inflammation and fibrosis. Nat. Rev. Nephrol. 2019. 15: 144–158.

Stewart, B. J., Ferdinand, J. R., Young, M. D., Mitchell, T. J., Loudon, K. W., Riding, A. M., Richoz, N. et al., Spatiotemporal immune zonation of the human kidney. Science. 2019. 365: 1461–1466.

Hoefel, G., Chen, J., Lavin, Y., Low, D., Almeida, F. F., See, P., Beaudin, A. E. et al., C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. Immunity. 2015. 42: 665–678.

Ide, S., Yahara, Y., Kobayashi, Y., Strauss, S. A., Ide, K., Watwe, A., Xu, Vanpala, S. et al., Yolk-sac-derived macrophages progressively expand in the mouse kidney with age. Elife. 2020. 9: e51756.

Liu, F., Dai, S., Feng, D., Qin, Z., Peng, X., Sakamuri, S., Ren, M. et al., Distinct fate, dynamics and niches of renal macrophages of bone marrow or embryonic origin. Nat. Commun. 2020. 11: 2280.

Casper, J., Schmitz, J., Bräsen, J. H., Khalifa, A., Schmidt, B. M. W., Einecke, G., Haller, H. et al., Renal transplant recipients receiving loop diuretic therapy have increased urinary tract infection rate and altered medullary macrophage polarization marker expression. Kidney Int. 2018. 94: 993–1001.

Bräsen, J. H., Khalifa, A., Schmitz, J., Dai, W., Einecke, G., Schwarz, A., Hallensleben, M. et al., Macrophage density in early surveillance biopsies predicts future renal transplant function. Kidney Int. 2017. 92: 479–489.

Helmeke, A., Hüsing, A. M., Gaedcke, S., Brauns, N., Balzer, M. S., Reinhardt, M., Hiss, M. et al., Peritoneal dialysate-range hypertonic glucose promotes T-cell IL-17 production that induces mesothelial inflammation. Eur J. Immunol. 2020. 50: 356–371.

Xu, K., Rosenstiel, P., Paragas, N., Hinze, C., Gao, X., Hui Shen, T., Werth, M. et al., Unique transcriptional programs identify subtypes of AKI. JASN. 2017. 28: 1729–1740.

Zhou, X., Ferraris, J. D., Cai, Q., Agrawal, A. and Burg, M. B. Increased reactive oxygen species contribute to high NaCl-induced activation of the osmoregulatory transcription factor TonEBP/OREBP. Am. J. Physiol.-Renal. Physiol. 2005. 289: F377–F385.

Higgins, J. P. T., Wang, L., Khambach, N., Montgomery, K., Mason, V., Vogelmann, S. U., Lemley, K. V. et al., Gene expression in the normal adult human kidney assessed by complementary DNA microarray. MBio. 2004. 15: 649–656.

Banwell, M. E., Tolley, N. S., Williams, T. J. and Mitchell, T. J. Regulation of human eotaxin-3/CCL26 expression: modulation by cytokines and glucocorticoids. Cytokine. 2002. 17: 317–323.

Collman, S., Kruger, M., Sawyer, G. and Hurst, R. Procyandin A2 modulates IL-4-induced CCL26 production in human alveolar epithelial cells. JIMIS. 2016. 17: 1888.

Hebenstreit, D., Luft, P., Schmiedlechner, A., Duschi, A. and Horejsihoek, J., SOCS-1 and SOCS-3 inhibit IL-4 and IL-13 induced activation of Eotaxin-3/CCL26 gene expression in HEK293 cells. Mol. Immunol. 2005. 42: 295–303.

Larose, M.-C., Chakir, J., Archambaut, A.-S., Joubert, P., Provost, V., Laviolette, M. and Flamand, N. Correlation between CCL26 production by human bronchial epithelial cells and airway eosinophils: involvement in patients with severe eosinophilic asthma. J. Allergy Clin. Immunol. 2015. 136: 904–913.

Nakayama, T., Watanabe, Y., Oslo, N., Higuchi, T., Shigeta, A., Mizuguchi, N., Katou, F. et al., Eotaxin-3/CC chemokine ligand 26 is a functional ligand for CX3CR1. J. Immunol. 2021. 185: 6472–6479.

Takao, K. and Miyakawa, T. Genomic responses in mouse models greatly mimic human inflammatory diseases. Proc. Natl. Acad. Sci. U S A. 2015. 112: 1167–1172.

Johnsen, M., Kubiczki, T., Yeroslavitz, A., Spith, M. R., Mörsdorf, J., Gobel, H., Bohl, K. et al., The integrated RNA landscape of renal preconditioning against ischemia-reperfusion injury. JASN. 2020. 31: 716–730.

Shi, C. and Pamer, E. G. Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. 2011. 11: 762–774.

Ogilvie, P., Paoletti, S., Clark-Lewis, I. and Uguccioni, M. Eotaxin-3 is a natural antagonist for CCR2 and exerts a repulsive effect on human monocytes. Blood. 2003. 102: 789–794.

Petkovic, V., Moghini, C., Paoletti, S., Uguccioni, M. and Gerber, B. J. Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5. A human chemokine with a regulatory role. Biol Chem. 2004. 279: 23357–63.

Müller, D. N., Wilck, N., Haase, S., Kleinewietfeld, M. and Liniker, R. A. Sodium in the microenvironment regulates immune responses and tissue homeostasis. Nat. Rev. Immunol. 2019. 19: 243–254.

Araburu, J. and López-Rodríguez, C. Regulation of inflammatory functions of macrophages and T lymphocytes by NFATs. Front. Immunol. 2019. 10: 535.

Engel, D. R., Maurer, J., Tittel, A. P., Weishelt, C., Cavlar, T., Schumak, B., Limmer, A. et al., CCR2 mediates homeostatic and inflammatory release of Gr1(high) monocytes from the bone marrow, but is dispensable for bladder infiltration in bacterial urinary tract infection. J. Immunol. 2008. 181: 5579–5586.

Hashimoto, S., Matsumoto, K., Kon, Y., Nakayama, T., Takeshita, I. and Horie, T. Hyperosmolarity-induced interleukin-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase. Am. J. Respir Crit. Care. Med. 1999. 159: 634–640.

Shapiro, L. and Dinarello, C. A. Osmotic regulation of cytokine synthesis in vitro. Proc. Natl. Acad. Sci. 1995. 92: 12230–12234.

Németh, Z. H., Deitch, E. A., Szabó, C. and Haskó, G. Hyperosmotic stress induces nuclear factor-κB activation and interleukin-8 production in human intestinal epithelial cells. Am. J. Pathol. 2002. 161: 987–996.

Ip, W. K. E. and Medzhitov, R. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. Nat. Commun. 2015. 6: 6931.

Shinkai, A., Yoshisue, H., Kolke, M., Shoji, E., Nakagawa, S., Saito, A., Takeda, T. et al., A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. J. Immunol. 1999. 163: 1602–1610.

Wohlforthova, M., Tycova, I., Honsova, E., Lodererova, A. and Vlkicky, O. Molecular patterns of subclinical and clinical rejection of kidney allograft: quantity matters. Kidney Blood Pres. Res. 2015. 40: 244–257.

El-Shazly, A. E., Doloriert, H. C., Bisig, B., Lefebvre, P. P., Delvenne, P. and Jacobs, N. Novel cooperation between CX3CL1 and CCL26 inducing NK cell chemotaxis via CX3CR1: a possible mechanism for NK cell infiltration of the allergic nasal tissue. Clin. Exp. Allergy. 2013. 43: 322–331.

Tian, W. and Cohen, D. M. Urea stress is more akin to EGF exposure than to hypertonic stress in renal medullary cells. Am. J. Physiol. Renal. Physiol. 2002. 283: F388–398.

McLaughlan, E. and Barth, J. H. An analysis of the relationship between serum cortisol and serum sodium in routine clinical patients. Pract. Laborat. Med. 2017. 8: 30–33.

Sailer, C. O., Wiedemann, S. J., Strauss, K., Schrader, I., Fenske, W. K. and Christ-Crain, M. Markers of systemic inflammation in response to osmotic stimulus in healthy volunteers. Endocr. Connect. 2019. 8: 1282–1287.
barrier function of the skin and boosts macrophage-driven host defense. Cell Metab. 2015. 21: 493–501.

48 Wenstedt, E. F. E., Verberk, S. G. S., Kroon, J., Neele, A. E., Baardman, J., Claessen, N., Pasaoğlu, Ö. T. et al., Salt increases monocyte CCR2 expression and inflammatory responses in humans. JCI Insight. 2019. 4: e130508.

49 Wilck, N., Matus, M. G., Kearney, S. M., Olesen, S. W., Forslund, K., Bartolomaeus, H., Haase, S. et al., Salt-responsive gut commensal modulates TH17 axis and disease. Nature. 2017. 551: 585–589.

50 Müller, S., Quast, T., Schröder, A., Hucke, S., Klotz, L., Jantsch, J., Gerzer, R. et al., Salt-dependent chemotaxis of macrophages. PLoS One. 2013. 8: e73439.

51 Minegishi, S., Luft, F. C., Titze, J. and Kitada, K. Sodium handling and interaction in numerous organs. Am. J. Hypertens. 2020. 33: 687–694.

52 Roy-Chowdhury, E., Brauns, N., Helmke, A., Nordlohne, J., Bräsen, J. H., Schmitz, J., Volkmann, J. et al., Human CD16+ monocytes promote a pro-atherosclerotic endothelial cell phenotype via CX3CR1–CX3CL1 interaction. Cardiovasc. Res. 2021. 117: 1510–1522.

53 Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., Sidiropoulos, K. et al., The reactome pathway knowledge base. Nucleic. Acids. Res. 2020. 48: D498–D503.

54 Cossarizza, A., Chang, H. D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., Andrä, I. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). Eur. J. Immunol. 2021. 51: 2708–3145.

Full correspondence: Dr. Sibylle von Vietinghoff, Nephrology Section, First Medical Clinic, University Clinic Bonn, Venusberg Campus 1, D-53127 Bonn, Germany.
Email: Sibylle.von_Vietinghoff@ukbonn.de

Received: 15/11/2021
Revised: 20/3/2022
Accepted: 6/5/2022
Accepted article online: 8/5/2022