Viral double-stranded RNA sensors induce antiviral, pro-inflammatory, and pro-apoptotic responses in human renal tubular epithelial cells

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Viral infection in the kidney is characterized by tubular injury induced directly by the virus and/or by cytotoxic lymphocytes. Previously, we found that human tubular epithelial cells express Toll-like receptor 3 (TLR3), melanoma differentiation–associated gene 5 (MDA5), and retinoic acid–inducible gene-I (RIG-I), all sensors of double-stranded RNA (dsRNA) and potent inducers of antiviral activity. Here, we demonstrate increased expression of these three dsRNA sensors in kidney transplant biopsies during cytomegalovirus or BK virus infection. In primary tubular epithelial cells, dsRNA sensor activation induced the production of pro-inflammatory TNF-α and antiviral IFN-β. Notably, dsRNA also enhanced the expression of pro-apoptotic proteins; however, dsRNA alone did not cause cell death due to the expression of anti-apoptotic proteins. The dsRNA sensitized tubular epithelial cells to apoptosis induced by an agonistic antibody against the Fas receptor (CD95), an apoptotic pathway that eliminates infected cells. These findings indicate that tubular epithelial cells require at least two signals to undergo apoptosis, which can help preserve tubular integrity even under inflammatory conditions. Thus, sensors of viral dsRNA promote antiviral, pro-inflammatory, and pro-apoptotic responses in tubular epithelial cells, which may orchestrate the control of viral infection in the kidney.

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Viral infection is a well-known complication after kidney transplantation, which can lead to graft dysfunction and may cause considerable morbidity and even mortality.[1] Cytomegalovirus (CMV), Epstein–Barr virus (EBV), and BK virus (BKV) are latently present in the majority of the adult population.[2–4] In immunocompromised patients, these viruses take the opportunity to reactivate. Moreover, they may be transferred with the donor transplant, generally resulting in a primary infection in recipients who lack immunity against the virus. Infection can elicit severe immune pathology leading to tubulointerstitial nephritis, which is characterized by infiltrating lymphocytes.[1,5,6] In addition, some viruses, such as BKV, have a lytic replication cycle inside renal tubular epithelial cells (TECs).[7]

Immune and non-immune cells can sense the presence of viruses with receptors that recognize virus-associated molecular patterns including nucleic acid structures.[8] Upon ligand binding, these pattern recognition receptors activate signaling pathways that inhibit viral replication and activate antiviral immune responses. A potent inducer of antiviral defense mechanisms is double-stranded RNA (dsRNA), which represents the genome of some viruses but is also an intermediate formed by most viruses during their replication cycle. Receptors that sense dsRNA are Toll-like receptor 3 (TLR3), which resides in the endosomal compartment and thus recognizes dsRNA taken up from the environment, and the cytoplasmic receptors melanoma differentiation–associated gene 5 (MDA5) and retinoic acid–inducible gene-I (RIG-I), which monitor viral replication within the cell.[9] Upon ligand binding, dsRNA receptors activate nuclear factor-κB (NF-κB), leading to the production of pro-inflammatory cytokines and interferon-regulating factors 3/7 (IRF3/7), which promote transcription of type I interferons (IFNs).[10] Type I IFNs consecutively induce IFN-stimulated genes, which regulate among others RNA stability and protein synthesis, transport, and turnover, thereby inhibiting viral replication.[11,12] Viruses have developed various

mechanisms to evade the human immune system, including the suppression of type I IFN responses and transcription, processing, and translation of host genes.[8]

If these cellular responses are not sufficient to control the virus, the only way to block viral replication is death of the infected cell. Recent studies indicate that dsRNA receptor signaling can promote cell death by inducing the expression and/or activation of various pro-apoptotic mediators.[13–18] Programmed cell death, also called apoptosis, can be induced in different ways during viral infection.[19] Triggering of PPRs, such as the dsRNA sensors mentioned above, can directly promote apoptosis in the infected cells. On the other hand, specialized cells of the immune system such as natural killer cells and cytotoxic T lymphocytes selectively eliminate virus-infected cells via two apoptotic pathways: the granule-exocytosis pathway, in which granzymes have a key role, and the death receptor–mediated pathway.[20,21] Activation of death receptors, such as CD95 (Fas), by their ligands, which are highly expressed on activated cytotoxic lymphocytes, is followed by assembly of the death-inducing signaling complex and cleavage of pro-caspase 8, resulting in the activation of effector caspases and nucleases.[22] This death receptor–induced cascade is known as the extrinsic pathway of apoptosis. In addition, apoptosis can be induced by various cellular stress signals that activate the mitochondrial or intrinsic pathway. The intrinsic apoptotic pathway is characterized by a disrupted balance between anti-apoptotic Bcl-2-like proteins and pro-apoptotic BH3-only proteins, leading to mitochondrial membrane permeabilization and formation of the apoptosome, a protein complex that activates effector caspases.[23] The extrinsic and intrinsic pathways are linked via the Bcl-2-interacting domain (BID), which promotes mitochondrial disintegration once it is cleaved by caspase 8.

We recently found that human TECs express TLR3, MDA5, and RIG-I,[24] suggesting that the cells are equipped to induce antiviral defense mechanisms upon recognition of viral dsRNA. Here, we aimed to study the function of viral dsRNA sensors in the human renal tubular epithelium. We observed that dsRNA receptor expression in kidney transplant biopsies was enhanced during infection with CMV and BKV and confirmed the expression of all three dsRNA receptors in the renal tubuli. Upon activation, TLR3, MDA5, and RIG-I enhanced the production of key pro-inflammatory and antiviral cytokines in primary TECs. Notably, we observed that dsRNA receptor ligands also enhanced the expression of several apoptotic mediators. We explored the mechanisms of apoptosis in TECs and found that dsRNA receptor triggering did not reduce viability by itself but sensitized the cells to CD95-mediated apoptosis.

RESULTS

Renal expression of TLR3, MDA5, and RIG-I is enhanced during viral infection

To study the expression of TLR3, MDA5, and RIG-I in the human kidney, we performed quantitative PCR analyses on snap-frozen kidney transplant biopsies obtained during active viral infection with CMV, EBV, and BKV. Transcript levels were compared with levels in biopsies from patients with stable renal function and normal histology (Figure 1a). We found that transcription of TLR3, MDA5, and RIG-I was significantly upregulated during infection with CMV and BKV. EBV infection was not associated with an apparent increase in dsRNA receptor mRNA. Immunohistochemical stainings were performed to identify which cell types expressed the dsRNA receptors (Figure 1b). TLR3 expression was low in stable and EBV biopsies, but TLR3-positive tubuli were present in all CMV biopsies and four of the seven BKV biopsies (Figure 1b and Supplementary Figure S1A online). MDA5 was broadly expressed in renal cells and infiltrating immune cells, yet its expression was most prominent in the proximal tubuli. We could not observe differences in MDA5 expression pattern and/or staining intensity between biopsies obtained during viral infection and stable controls. In line with the transcript levels, tubular RIG-I expression seemed enhanced in biopsies obtained during infection with CMV and BKV, as analyzed by semiquantitative scoring (Supplementary Figure S1B online). Next to the tubular epithelium, endothelial cells and graft-infiltrating leukocytes expressed abundant levels of RIG-I. In summary, CMV and BKV infection are associated with increased renal dsRNA receptor expression, and TLR3, MDA5, and RIG-I are expressed by the tubular epithelium during viral infection.

Viral dsRNA promotes the production of pro-inflammatory and antiviral cytokines in primary TECs

dsRNA receptor triggering is known to activate IRF3/7 and NF-κB, leading to the production of, respectively, antiviral and pro-inflammatory cytokines.[9,25,26] To address the ability of dsRNA receptors to evoke antiviral immune responses in TECs, we stimulated primary cells with known dsRNA receptor ligands. Poly(I:C) is a synthetic dsRNA molecule that activates both TLR3 and MDA5. Although administration of the ligand outside the cell predominantly triggers TLR3, transfection is commonly used to activate MDA5.[13,27] We used Fugene HD to deliver poly(I:C) or 5′triphosphate RNA (3pRNA), a specific RIG-I ligand, to the cytoplasm. Activation of the dsRNA sensors dose dependently induced transcription of IFN-β, the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and the chemokine IFN-inducible protein 10 (Figure 2a and Supplementary Figure S2A online). IFN-α mRNA was only detectable after stimulation with high amounts of 3pRNA, and culture supernatants contained no detectable amount of IFN-α protein (ELISA data not shown). Notably, activation of the cytoplasmic sensors MDA5 and RIG-I most prominently induced IFN-β mRNA and protein expression, whereas TLR3 was a more potent inducer of TNF-α production (Figures 2b and c).

As a model for viral infection, we used influenza A, which is known to activate TLR3 and RIG-I.[28] Influenza enhanced the transcription of TLR3, MDA5, IFN-β, and TNF-α

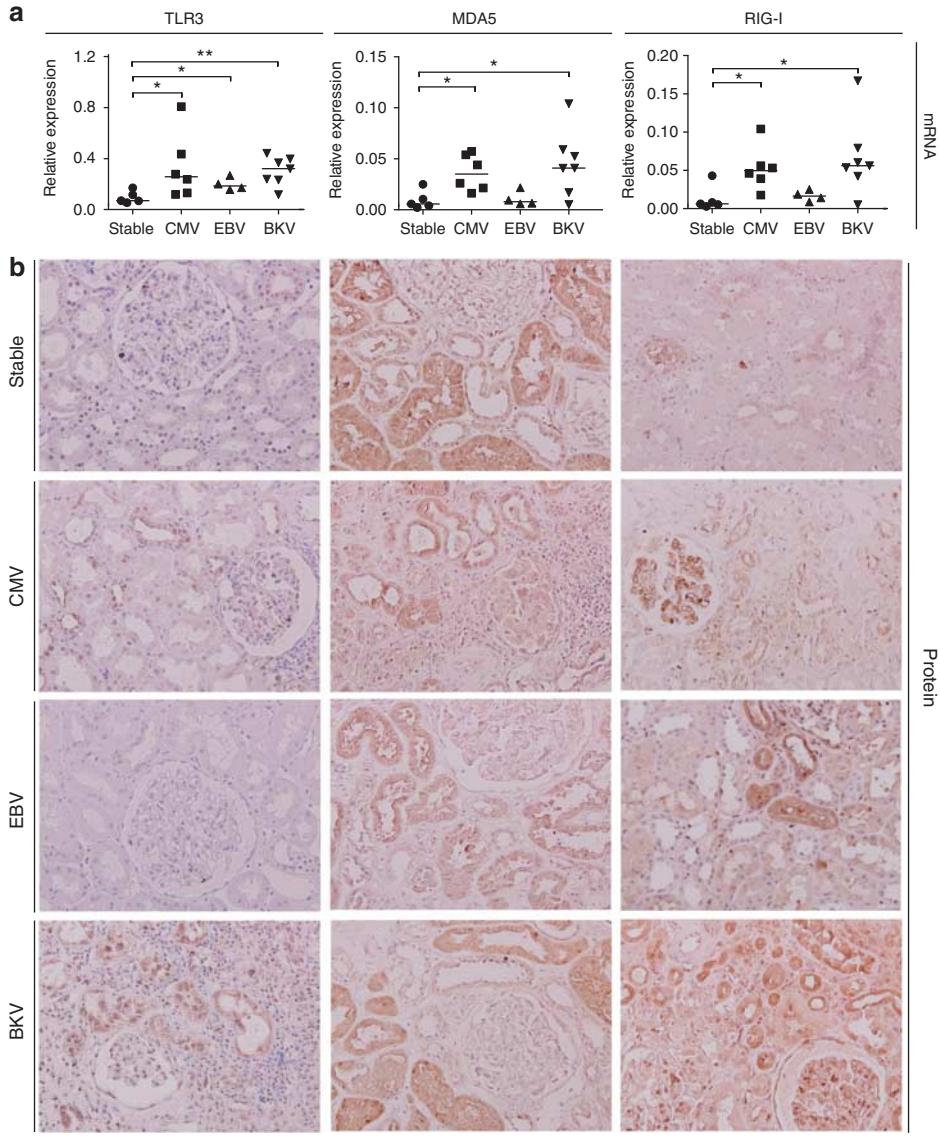

Figure 1 | Double-stranded RNA (dsRNA) receptor expression in the kidney is increased during viral infection. Expression of the dsRNA receptors in kidney transplant biopsies obtained from patients with a stable renal function and normal histology (stable, $n = 5$) or during infection with cytomegalovirus (CMV, $n = 6$), Epstein–Barr virus (EBV, $n = 4$), or BK virus (BKV, $n = 7$). (**a**) Transcript levels of Toll-like receptor 3 (TLR3), melanoma differentiation–associated gene 5 (MDA5), and retinoic acid–inducible gene-I (RIG-I) determined by quantitative PCR in snap-frozen biopsies (scatter, median). Expression levels in virus-infected biopsies were compared with stable biopsies (Mann–Whitney *U*-tests, $^{*}P < 0.05$, $^{**}P < 0.01$). (**b**) Representative pictures of formalin-fixed, paraffin-embedded biopsies stained immunohistochemically for TLR3, MDA5, or RIG-I.

(Supplementary Figure 2B and Figure 2D). Viable virus did not promote the secretion of either IFN-β or TNF-α (Figure 2e and f), suggesting that influenza induces the expression of viral proteins that interfere with proper translation and/or secretion of host cytokines.[8,29,30] We observed that the induction of TLR3, MDA5, and IFN-β mRNA, but not that of TNF-α transcripts, was abolished after heat inactivation of the virus. TNF-α protein was abundantly produced in TECs treated with heat-inactivated virus. These findings imply that genomic RNA or protein present in heat-inactivated influenza particles is recognized by TLR3 or other pattern recognition receptors that mediate TNF-α expression. On the other hand, heat-inactivated influenza did not induce transcription and secretion of IFN-β. Thus, viral replication seems to be required for triggering of the cytoplasmic dsRNA sensors, which in our hands were the main inducers of IFN-β in TECs. In summary, viral dsRNA receptors regulate cytokine production in TECs. Antiviral cytokines were in particular produced in response to dsRNA mimics delivered to the cytoplasm, whereas extracellular dsRNA strongly enhanced the expression of pro-inflammatory cytokines.

dsRNA receptor activation renders TECs sensitive to CD95-mediated apoptosis

Several studies have shown that dsRNA receptor signaling can induce death in a cell type–dependent manner.[13–18] We

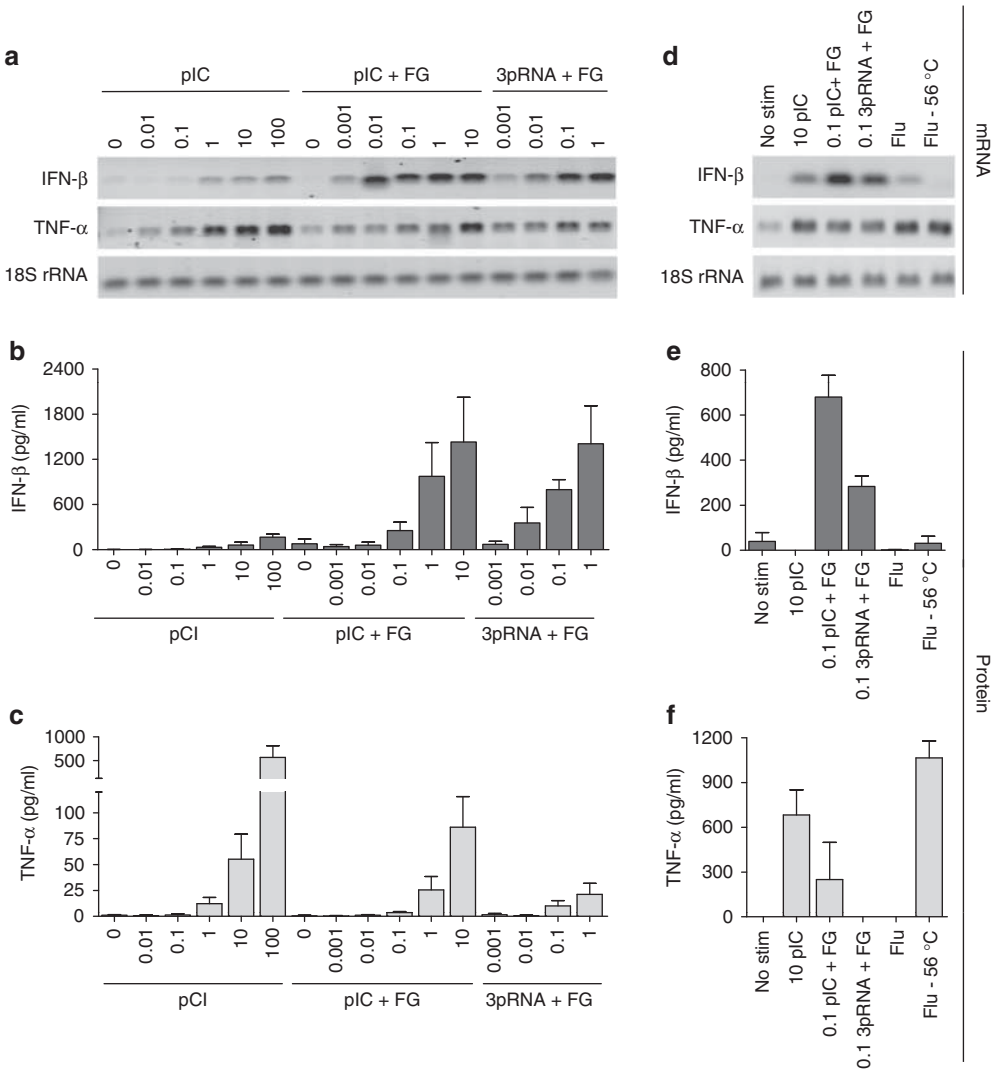

Figure 2 | Activation of viral double-stranded RNA (dsRNA) receptors promotes the production of pro-inflammatory and antiviral cytokines in tubular epithelial cells (TECs). Primary TECs were stimulated for 16 h with poly(I:C) (pIC, 0.01–10 μg/ml), poly(I:C) and Fugene (pIC + FG, 0.001–10 μg/ml), 5′triphosphate RNA (3pRNA) and Fugene (3pRNA + FG, 0.001–1 μg/ml), influenza A (Flu), heat-inactivated influenza (30 min at 56 °C), or kept unstimulated (no stim). (**a**, **d**) Transcript levels of interferon (IFN)-β and tumor necrosis factor (TNF)-α determined by PCR. The PCR bands shown are representative of at least three experiments. Protein levels of IFN-β (**b**, **e**) and TNF-α (**c**, **f**) analyzed by ELISA in culture supernatants of TECs stimulated for 24 h as described above (mean ± s.e.m., $n = 3$).

wondered whether dsRNA receptor triggering could promote cell death in human TECs, and, if so, which apoptotic pathways were involved. Annexin V and propidium iodide staining showed that the great majority of TECs stimulated with dsRNA analogs remained viable (Figure 3a). CD95 ligand–bearing cytotoxic lymphocytes can clear virus-infected cells. We mimicked this immune cell interaction with an agonistic antibody against CD95 (CH11). In agreement with others,[31,32] we found that TECs were resistant to CD95-mediated apoptosis even at high concentrations of CH11 (4 μg/ml; Figure 3a and Supplementary Figure S3A online). Interestingly, dsRNA receptor activation induced CD95 expression (Figure 3b and data not shown) and rendered TECs sensitive to CD95-mediated apoptosis in a dose-dependent manner (Figure 3a, c and d). Extracellular administration of 10 μg/ml poly(I:C) and intracellular delivery of 0.1 μg/ml poly(I:C) or 3pRNA resulted in comparable percentages of apoptotic TECs (Figure 3e); therefore, we used these concentrations henceforth. TECs infected with influenza underwent apoptosis after CD95 ligation as well (Figure 3f), demonstrating that virus can activate a response similar to that of the tested dsRNA receptor ligands. Activation of other TLRs that recognize viral single-stranded RNA (TLR7), DNA (TLR9), or bacterial cell wall (TLR2) or membrane (TLR4) compounds did not render TECs sensitive to CD95-mediated apoptosis (Supplementary Figure S3B online).

To analyze which signaling cascades, known to be induced by dsRNA receptor triggering, are responsible for the increased sensitivity to CD95-mediated apoptosis, we studied

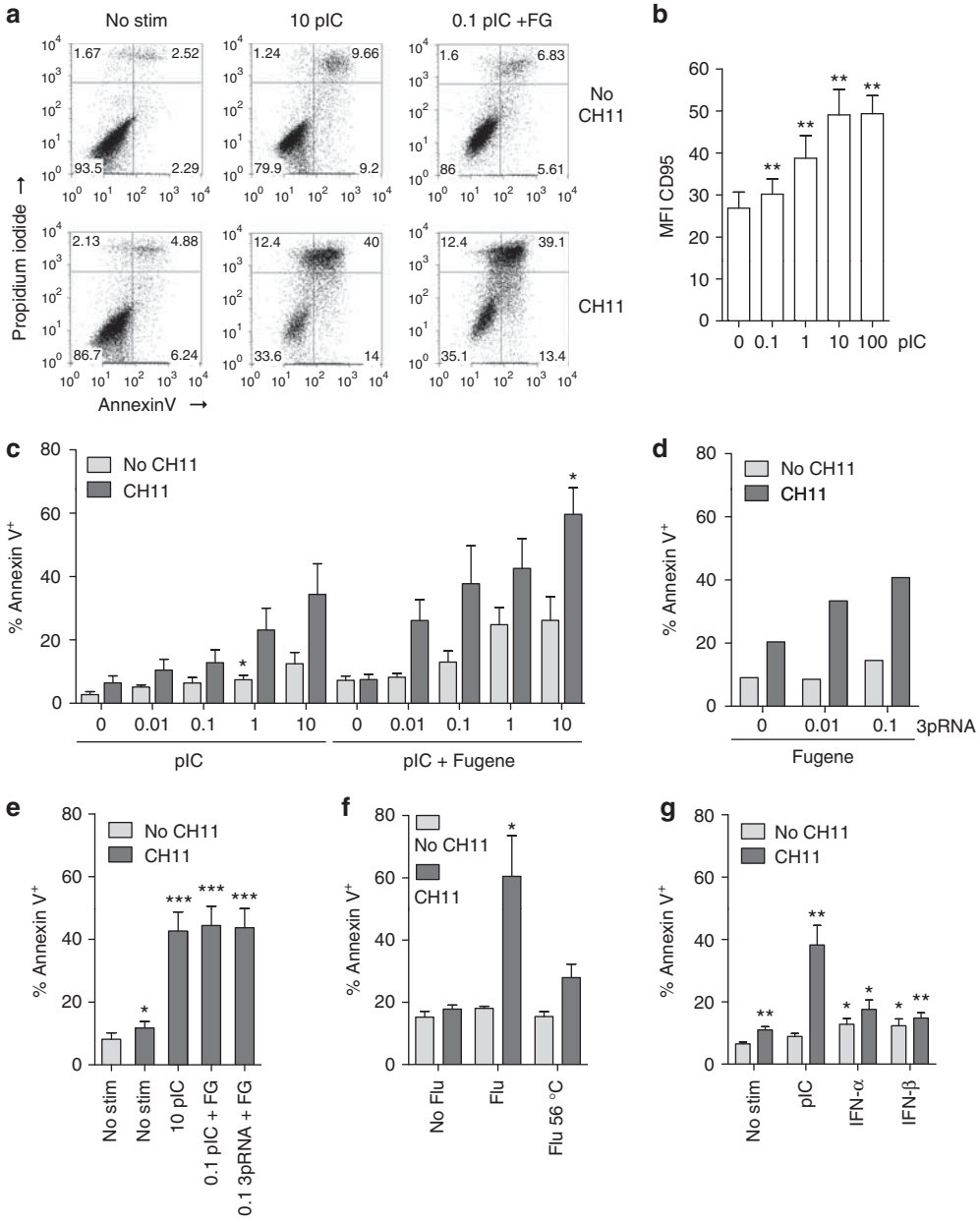

Figure 3 | Triggering of double-stranded RNA (dsRNA) receptors renders tubular epithelial cells (TECs) sensitive to CD95-mediated cell death. Primary TECs were stimulated with 0.01–10 μg/ml poly(I:C) (pIC), 0.01–10 μg/ml poly(I:C) plus Fugene (pIC + FG), or 0.01–0.1 μg/ml 5′triphosphate RNA (3pRNA) plus Fugene (3pRNA + FG) to activate Toll-like receptor 3, melanoma differentiation–associated gene 5, and retinoic acid–inducible gene-I, or kept unstimulated (no stim). After 16 h, an agonistic IgM antibody against CD95 (CH11, 2 μg/ml) was added for 24 h. The percentage of apoptotic TECs was determined by annexin V and propidium iodide staining and flow cytometry. (**a**) Representative fluorescence-activated cell sorting plots of stimulated TECs. (**b**) CD95 expression on TECs stimulated with poly(I:C) (0.1–100 μg/ml) depicted as mean fluorescent intensity (mean ± s.e.m., $n = 4$). (**c**) Percentage of annexin V^+ TECs after stimulation with increasing amounts of poly(I:C) (mean ± s.e.m., $n = 3$). (**d**) Percentage of annexin V^+ TECs after stimulation with 3pRNA (mean, $n = 2$). (**e**) Apoptosis in TECs after dsRNA receptor stimulation and CD95 ligation as described above (mean ± s.e.m., $n = 12$). (**f**) Percentage of annexin V^+ cells (mean ± s.e.m., $n = 3$) after infection with influenza A (Flu) or heat-inactivated virus (30 min at 56 °C) for 16 h, followed by 24 h incubation with CH11. (**g**) Percentage of Annexin V^+ TECs after stimulation with poly(I:C) (10 μg/ml), interferon (IFN)-α (1000 U/ml), or IFN-β (1000 U/ml) and anti-CD95 IgM (CH11) (mean ± s.e.m., $n = 6$). Values measured after stimulation or infection were compared with values found in resting TECs with unpaired *t*-tests. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the involvement of several key mediators. In TECs, apoptosis appeared to be NF-κB independent, as sensitivity to CD95-mediated apoptosis was not induced by other NF-κB activators, such as IL-1β and TLR2/4 ligands (Supplementary Figure S3B online), and apoptosis induced by dsRNA and CD95 was not affected by the NF-κB inhibitor Bay11-7082 (Supplementary Figure S4A online). Type I IFNs, which are transcribed upon dsRNA receptor–induced IRF3/7 activation,

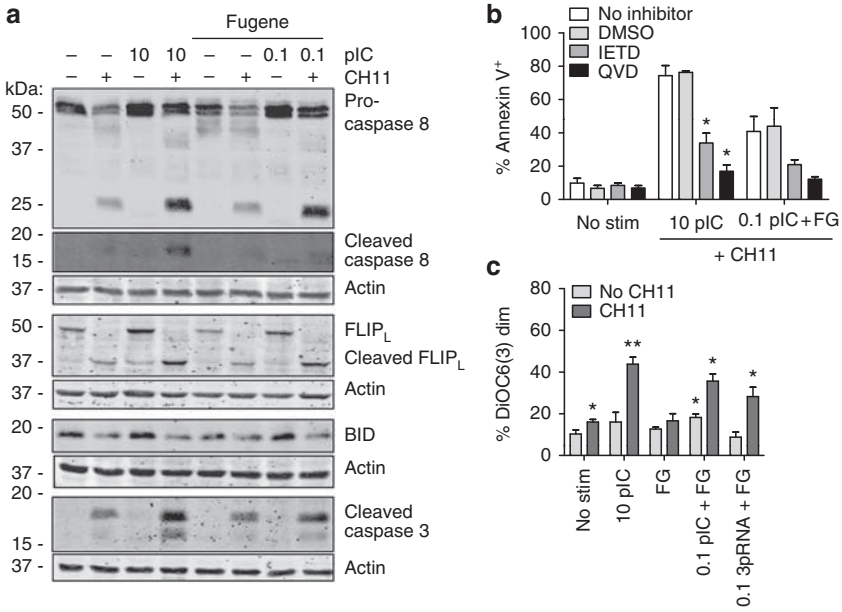

Figure 4 | Intrinsic and extrinsic apoptotic pathways are involved in double-stranded RNA (dsRNA) receptor– and CD95-mediated death of tubular epithelial cells (TECs). (**a**) Primary TECs were stimulated with 10 μg/ml poly(I:C) (pIC) or 0.1 μg/ml poly(I:C) plus Fugene (pIC + FG). After 16 h, anti-CD95 IgM (CH11, 2 μg/ml) was added for an additional 6 h. Expression and cleavage of caspase 8, FLICE-like inhibitory protein (FLIP), Bcl-2-interacting domain (BID), and caspase 3 was determined by western blot analysis. The blots are representative of at least three experiments. (**b**) Caspase activity was blocked with the caspase 8 inhibitor Z-IETD-FMK (IETD, 20 μmol/l) or the pan-caspase inhibitor Q-VD-OPH (QVD, 20 μmol/l) added 30 min before stimulation with 10 μg/ml poly(I:C) or 0.1 μg/ml poly(I:C) + Fugene (40 h), or to unstimulated cells (no stim). CD95 antibody (CH11) was added during the last 24 h of the stimulation. The bars represent the percentage of annexinV$^+$ cells (mean ± s.e.m., $n = 3$) measured by flow cytometry. (**c**) TECs were stimulated for 16 h with 10 μg/ml poly(I:C), 0.1 μg/ml poly(I:C) and Fugene, or 0.1 μg/ml 5′triphosphate RNA (3pRNA) and Fugene; subsequently, CD95 was activated (CH11, 24 h). The percentage of DiOC6(3)-low TECs, representing cells that have lost their mitochondrial membrane potential, is depicted (mean ± s.e.m., $n = 3$). Percentages of death cells after dsRNA receptor and/or CD95 stimulation were compared with the percentage found in resting TECs with paired *t*-tests (*$P < 0.05$, **$P < 0.01$). DMSO, dimethyl sulfoxide.

can induce or enhance apoptosis.[12,33,34] IFN-α and IFN-β slightly reduced TEC viability but did not render the cells sensitive to CD95-mediated apoptosis, indicating that type I IFNs were not the main mediators of cell death (Figure 3g). In summary, dsRNA sensitizes TECs to CD95-mediated cell death independently of type I IFNs and NF-κB.

The extrinsic and intrinsic apoptotic pathways are activated in TECs upon stimulation of dsRNA receptors and CD95

Triggering of CD95 promotes auto-cleavage of pro-caspase 8, leading to the activation of effector caspase 3, both directly and via the mitochondrial route initiated by BID cleavage.[22] To unravel the mechanism of CD95- and dsRNA-mediated apoptosis, we analyzed the activation of the extrinsic and intrinsic apoptosis pathways. CD95 stimulation alone did induce cleavage of caspase 8, its inhibitor FLIP, and BID, leading to some active caspase 3 (Figure 4a). By itself, dsRNA did not promote caspase activation or cleavage of FLIP and BID but did enhance expression of pro-caspase 8. Combined activation of dsRNA receptor and CD95 facilitated the generation of active caspase 3, both the 19-kDa form and the 17-kDa form. Apoptosis was blocked largely by the pan-caspase inhibitor Q-VD-OPH and partially by the caspase 8 inhibitor Z-IETD-FMK, indicating involvement of caspase 8 (Figure 4b). Incomplete inhibition of cell death in the presence of Z-IETD-FMK suggested involvement of the mitochondrial pathway in dsRNA- and CD95-mediated apoptosis. Indeed, the percentage of TECs with a reduced DiOC6(3) staining, representing cells with a loss of mitochondrial membrane potential, was increased after triggering dsRNA receptors and CD95 (Figure 4c). It is noteworthy that viral dsRNA did not render TECs sensitive to apoptosis induced by two other death receptor ligands—TNF-α and TRAIL—demonstrating that our findings are specific for CD95 (Supplementary Figure S4B online). Thus, CD95 and dsRNA trigger cell death, which is caspase dependent and involves activation of both the extrinsic and intrinsic pathways of apoptosis.

dsRNA receptors regulate the expression of pro- and anti-apoptotic mediators

To address how dsRNA receptor activation sensitizes TECs to CD95-mediated apoptosis, we analyzed the expression of genes involved in cell death by multiplex ligation–dependent probe amplification.[35] In response to dsRNA, TECs rapidly increased transcription of the pro-apoptotic proteins Noxa and Puma and the inhibitors of apoptosis cIAP2 and NAIP (Figure 5a and b). At the protein level, the induction of Noxa and Puma coincided with increased expression levels of their anti-apoptotic binding partner Mcl-1 (Figure 5c). Notably,

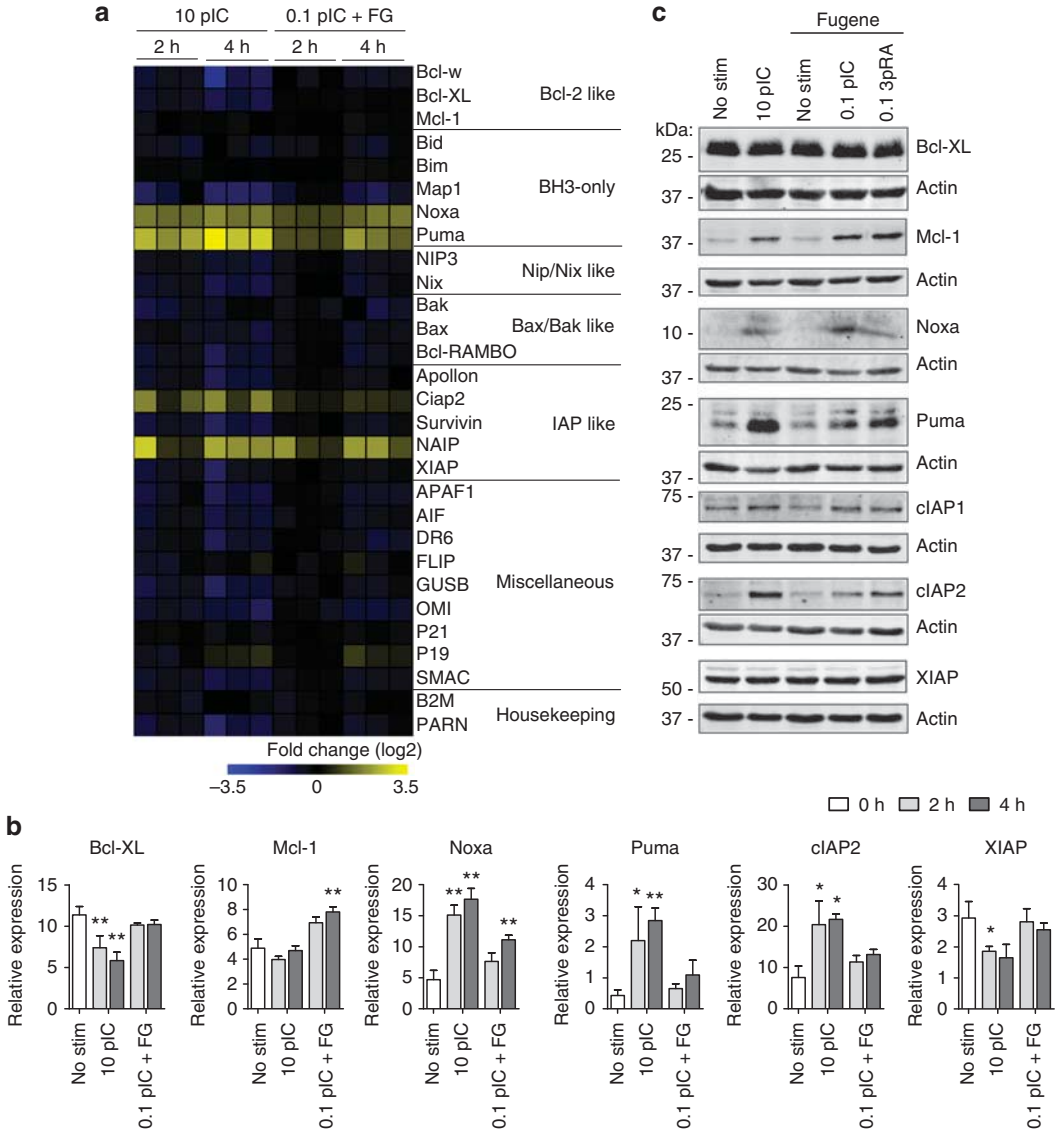

Figure 5 | Activation of double-stranded RNA (dsRNA) receptors promotes the expression of pro-apoptotic BH3-only and anti-apoptotic IAP family members. Primary tubular epithelial cells (TECs) were stimulated with 10 μg/ml poly(I:C) (pIC) or 0.1 μg/ml poly(I:C) and Fugene (pIC + FG) for 2 or 4 h, or kept unstimulated (no stim). Subsequently, mRNA expression levels of genes that mediate apoptosis were determined by multiplex ligation-dependent probe amplification (MLPA). (**a**) Heat-map of fold change (log2) in gene expression compared with unstimulated TECs. Each square represents a single measurement ($n = 3$). Genes that are upregulated are depicted in yellow, and genes that are downregulated are depicted in blue. (**b**) Transcription levels of Bcl-XL, Mcl-1, Noxa, Puma, cIAP2, and XIAP measured by MLPA are depicted as relative expression (arbitrary values, mean ± s.e.m., $n = 3$). Values were compared with unstimulated cells with paired *t*-tests (*$P < 0.05$, **$P < 0.01$). (**c**) Analysis of pro- and anti-apoptotic genes by western blot analysis in TECs stimulated for 24 h with 10 μg/ml poly(I:C), 0.1 μg/ml poly(I:C) and Fugene, and 0.1 μg/ml 5′triphosphate RNA (3pRNA). Blots are representative of at least three different experiments.

the protein expression pattern observed after stimulation of TLR3, MDA5, and RIG-I were largely comparable.

Next to Noxa and Puma, dsRNA receptor activation induced the expression of several members of the IAP family (Figure 5), which inhibit death receptor– and caspase-mediated apoptosis.[36] To study the influence of IAPs in limiting CD95- and dsRNA-mediated apoptosis, we used a Smac mimetic, compound A, which promotes proteasomal degradation of IAPs (Figure 6a). Degradation of IAPs rendered TECs sensitive to CD95-mediated apoptosis in a dose-dependent manner (Figure 6b). Furthermore, we found that TECs died after the addition of compound A and dsRNA, demonstrating that dsRNA receptor could indeed mediate apoptosis provided that IAPs were blocked. Combination of dsRNA receptor and CD95 activation and compound A resulted in an even more pronounced cell death (Figure 6c and Supplementary Figure S4C online). IAP antagonists, such as compound A, can promote apoptosis via NF-κB-stimulated production of TNF-α in sensitive tumor cell lines.[37] In TECs, TNF-α–mediated apoptosis did not have a prominent role, as compound A did not enhance TNF-α secretion (Supplementary Figure S4D online), and TNF-α by

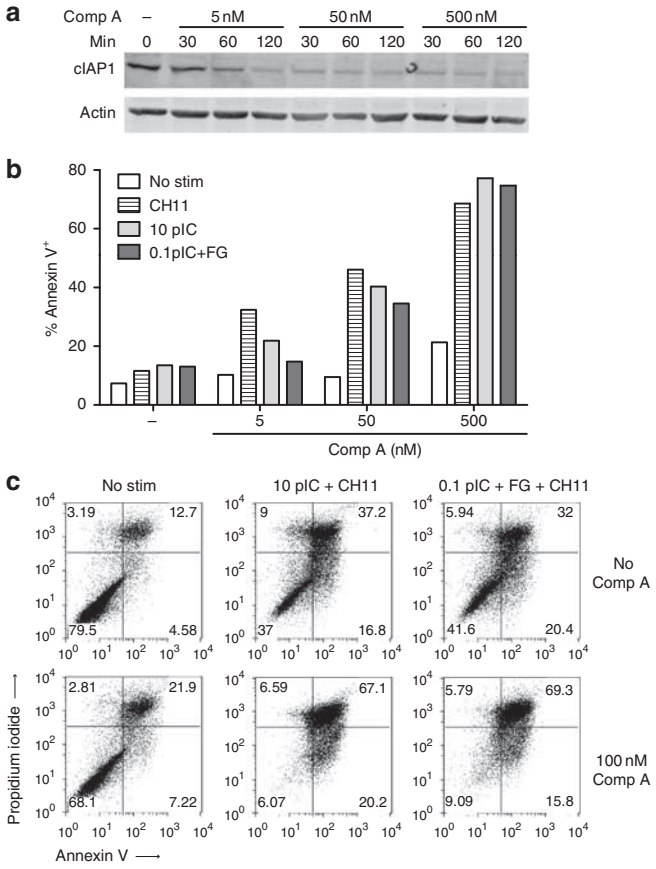

Figure 6 | IAPs control apoptosis induced by CD95 or double-stranded RNA (dsRNA) receptor activation in tubular epithelial cells (TECs). (**a**) Expression of cIAP1 in TECs stimulated with compound A (5–500 nmol/l, 30–120 min) analyzed by western blotting. (**b**) TECs incubated with compound A (30 min) were stimulated with anti-CD95 IgM (CH11, 2 μg/ml, 24 h), poly(I:C) (pIC, 40 h), poly(I:C) with Fugene (pIC + FG, 40 h), or kept unstimulated (no stim). Apoptosis was analyzed by flow cytometry; the bars represent the percentage of annexin V^+ TECs (mean, $n = 2$). (**c**) Fluorescence-activated cell sorting plots of TECs stimulated as described above stained with annexin V and propidium iodide, representative of two experiments.

itself or in combination with dsRNA did not enhance apoptosis (Supplementary Figure S4B online). Together, these findings indicate that TECs are protected against various apoptotic stimuli because of the expression of anti-apoptotic proteins of the Bcl-2-like and IAP families.

DISCUSSION

Recently, we found that human renal TECs express the dsRNA receptors TLR3, MDA5, and RIG-I.[24] We here demonstrate that the expression of these receptors in human kidney transplants is enhanced during viral infection. Each receptor had a unique distribution, yet all could be detected in the renal tubuli. In agreement, we found that in primary TECs activation of TLR3, MDA5, and RIG-I induced the production of antiviral and pro-inflammatory cytokines. Furthermore, we observed that viral dsRNA induced a pro-apoptotic program that sensitized TECs to CD95-mediated apoptosis. Both the extrinsic and intrinsic apoptotic pathways were activated in response to dsRNA and CD95 ligation. In a broader context, this suggests that upon encounter of viral dsRNA or dsRNA from any other source TECs become susceptible to CD95-mediated cell death.

Several findings suggest that dsRNA receptors are involved in the recognition of DNA viruses that may reactivate in immunosuppressed kidney transplant recipients. CMV has been shown to activate TLR3 in THP-1 cells[38] and to induce a type I IFN response independent of TLR3 in dendritic cells.[39] EBV-encoded small RNAs are known ligands for TLR3 and RIG-I.[40,41] We observed that tubular dsRNA receptor expression was hardly increased during EBV infection. This might be due to the small sample size, but more likely reflects the low percentage of tubuli infected by EBV. EBV predominantly targets B cells,[42] whereas CMV is promiscuous,[43,44] and BKV infects epithelial cells of the urogenital tract.[5] Furthermore, EBV only rarely causes tubulitis and/or tubular injury in contrast to the other viruses.[45,46]

RIG-I was highly expressed in graft-infiltrating lymphocytes and endothelial cells, which can be infected by CMV.[43] In glomerular endothelial cells, dsRNA-induced antiviral responses are mediated by RIG-I.[47] Endothelial expression of the cytoplasmic sensor is induced by poly(I:C) and IFN-γ,[48,49] a cytokine abundantly produced by CMV-specific T cells.[50] Together, these findings suggest that endothelial RIG-I expression found in renal biopsies may result from viral infection.

A recent study by Ribeiro and co-workers showed that BKV infection promoted the transcription of TLR3, RIG-I, and inflammatory mediators in renal collecting duct cells,[51] suggesting that the virus can trigger one or more dsRNA receptors as well. Furthermore, the authors observed enhanced transcription of TLR3, but not of RIG-I, in biopsies with BKV-associated nephropathy as compared with donor biopsies. Our study confirms and extends these observations by demonstrating that TLR3, MDA5, and RIG-I mRNA levels were increased during BKV and CMV infection. We used biopsies obtained from stable transplant patients without rejection or infection as a control, which could explain the discrepancy in RIG-I transcription. BKV-associated nephropathy and acute cellular rejection are remarkably similar, based on immunohistological analysis and gene expression profile.[52] Indeed, TLR3 and RIG-I transcription in the kidney seems enhanced during acute rejection as well,[51,53] suggesting that dsRNA receptor expression is a general characteristic of renal inflammation. Microarray analyses showed that BKV infection increases cell cycle and apoptosis gene expression in TECs and endothelial cells.[54,55] Key viral defense genes were, however, not induced by BKV, which might be the result of immunosuppressive mechanisms developed by the virus. In line with this hypothesis, BKV-induced transcription of IL-6 and IL-8 in collecting duct cells occurred only within the first 6 h.[51]

It is increasingly recognized that dsRNA receptor activation can promote apoptosis. First of all, activation of MDA5 and RIG-I has been shown to trigger the intrinsic apoptotic pathway via enhanced Noxa expression in various cancer cells.[13,14,56] Second, RIG-I stimulation has been demonstrated to promote direct interaction of IRF-3 with Bax followed by loss of mitochondrial membrane potential.[15] In mesangial cells and some myeloma cell lines, apoptosis is mediated by type I IFNs, which are produced in response to poly(I:C).[57,58] Finally, TLR3 and its adaptor TRIF directly induced activation of caspase 8 in keratinocytes.[18] Here, we demonstrate for the first time a role of dsRNA receptors in the induction of apoptosis in the kidney and provide some insights into the mechanisms involved. Figure 7 schematically depicts the influence of dsRNA receptor triggering on the extrinsic and intrinsic pathways of apoptosis in TECs.

In agreement with previous studies, we found that dsRNA receptor activation induced expression of the pro-apoptotic BH3-only proteins Noxa and Puma.[13,14,18] Noxa and Puma inhibit the anti-apoptotic Bcl-2-like proteins. Puma is a promiscuous inhibitor, whereas Noxa specifically interacts with Mcl-1, A1, and probably Bcl-XL.[59–61] In primary TECs, Noxa and Puma induction appeared to coincide with an increased amount of their antagonist Mcl-1. Furthermore, Bcl-XL, another Bcl-2-like protein that can protect against MDA5- or RIG-I-mediated apoptosis,[13] was highly expressed in resting and stimulated TECs. Unfortunately, we could not address the exact contribution of dsRNA-induced Noxa and Puma to apoptosis, as small interfering and short hairpin RNA molecules seemed to enhance apoptosis in primary TECs (data not shown).

TECs constitutively expressed high levels of XIAP, a known inhibitor of the apoptosome.[36] dsRNA induced the expression of cellular IAPs, which can inhibit death receptor–mediated apoptosis but also have an important role in cell signaling.[36,62] We found that dsRNA did not promote activation of caspase 8 and/or caspase 3. However, in the absence of IAPs, poly(I:C) did induce cell death in a caspase

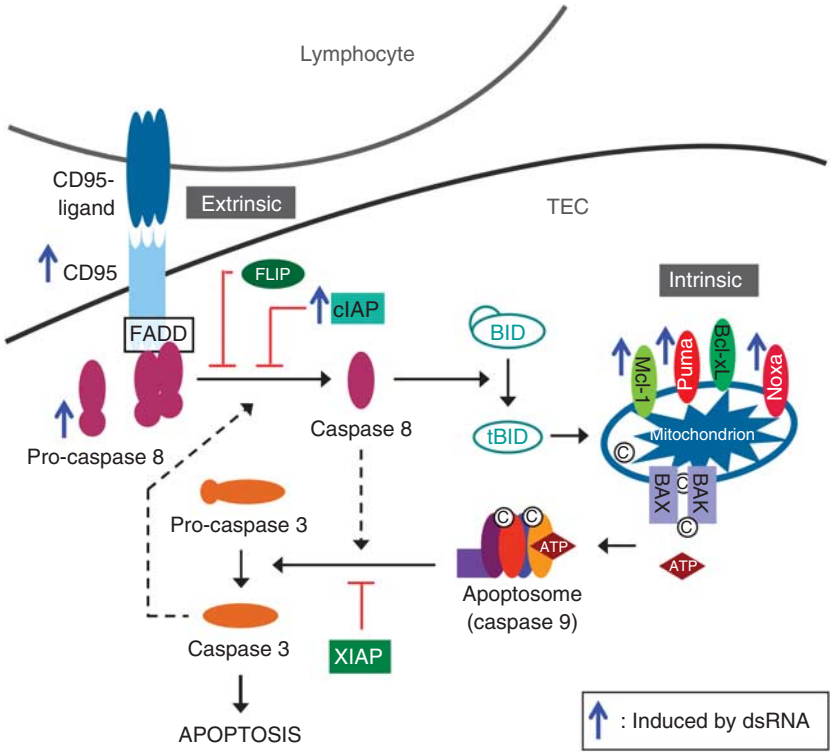

Figure 7 | Model for double-stranded RNA (dsRNA)- and CD95-mediated apoptosis in tubular epithelial cells (TECs). TECs are type II cells in which death receptor–mediated apoptosis requires activation of the intrinsic apoptosis pathway. CD95/CD95 ligand interaction induces the formation of the death-inducing signaling complex (DISC), which includes the adaptor CD95–associated death domain (FADD) and pro-caspase 8 leading to autocleavage of caspase 8. Active caspase 8 can in turn cleave the caspase 8 inhibitor FLICE-like inhibitory protein (FLIP) and Bcl-2-interacting domain (BID). The truncated form of BID (tBID) translocates to the mitochondrial membrane where it disrupts the balance between Bcl-2-like proteins, such as Bcl-XL and Mcl-1, and BH3-only proteins, such as Noxa and Puma. Anti-apoptotic Bcl-2-like proteins prevent interaction between Bak and Bax, which is essential for mitochondrial outer membrane permeabilization and cytochrome C release. Disruption of mitochondrial integrity leads to the formation of the apoptosome, a protein complex in which caspase 9 is activated in an ATP-dependent manner. The apoptosome promotes the activation of effector caspases such as caspase 3, leading to cell death. XIAP and cIAP1/2 block apoptosis by inhibiting caspase 3 and 8 activation. We found that expression of these inhibitors prevented death in TECs exposed to a single apoptotic stimulus such as CD95 or dsRNA. On the basis of these findings, we propose that the activation of viral dsRNA sensors sensitizes TECs to the attack of CD95 ligand–expressing cytotoxic lymphocytes by enhancing the expression of the death receptor CD95, pro-caspase 8, Puma, Noxa, and Mcl-1. The effect of dsRNA on expression levels of various mediators of the extrinsic and intrinsic apoptotic pathways is indicated by blue arrows.

8–dependent manner (Supplementary Figure S4C online). Thus, it seems likely that TECs are protected against dsRNA receptor–mediated apoptosis due to expression of Bcl-2-like and IAP family members.

It is known that despite constitutive CD95 expression TECs are resistant to CD95-mediated apoptosis.[31,32] In agreement herewith, we found that CD95 activation by itself did not result in cell death. CD95 ligation did induce the cleavage of pro-caspase 8, its inhibitor FLIP, and BID, demonstrating that TECs are so-called type II cells in which caspase 3 is not directly cleaved by caspase 8 in response to CD95 ligation. Type II cells express high levels of XIAP and require amplification of the apoptotic signal via the intrinsic apoptotic pathway.[63] Indeed, we found that CD95 triggering induced apoptosis in the presence of an IAP antagonist, which was almost completely caspase 8–dependent. This suggests that IAP inhibitors can turn type II cells, such as TECs, into type I cells. Moreover, we found that dsRNA receptor activation rendered TECs sensitive to CD95-mediated apoptosis. In line with these findings, other studies have demonstrated that human and murine TECs require a second signal such as inflammatory cytokines to become sensitive to apoptotic stimuli, such as CD95 ligand and TRAIL.[31,64–66] It is noteworthy that the activation of dsRNA receptors did not enhance sensitivity to apoptosis induced by the death receptor ligands TNF-α and TRAIL, suggesting that viral dsRNA specifically sensitizes TECs for CD95-mediated apoptosis.

In summary, our data demonstrate that TLR3, MDA5, and RIG-I contribute to antiviral immune responses in the human kidney and suggest that the dsRNA receptors have a role in the recognition of CMV and BKV, latent viruses that regularly reactivate in kidney transplant recipients. Furthermore, we show that dsRNA receptor activation induces a pro-apoptotic program, which sensitizes TECs to CD95-mediated apoptosis. The net balance in expression levels of pro- and anti-apoptotic mediators such as Noxa, Puma, Bcl-2-like family members, and IAPs appears to determine the sensitivity of TECs to various apoptotic stimuli, thereby defining whether infected cells are sensitive to CD95-mediated cell death leading to viral clearance or whether they survive allowing viral persistence.

MATERIALS AND METHODS

Kidney transplant patients

We included 22 kidney transplant biopsies performed by protocol or upon clinical indication. Viral infection was diagnosed by positive peripheral blood PCR, specific antibody conversion, and/or immunohistochemistry. Supplementary Table S1 online depicts the patient characteristics. Written informed consent was obtained from all study patients, and the study was approved by the Institutional Ethics Committee of the Academic Medical Center.

mRNA measurements

dsRNA receptor expression, determined by quantitative PCR in mRNA isolated from snap-frozen renal biopsies, was normalized to a control sample and glyceraldehyde 3-phosphate dehydrogenase. Cytokine expression in stimulated TECs was determined by semiquantitative PCR. Primer characteristics are depicted in Supplementary Table S2 online.

Immunohistochemistry

Immunohistochemical stainings were performed on formalin-fixed paraffin-embedded tissue slides with a monoclonal mouse IgG1 antibody against TLR3 (5 μg/ml, clone 40C1285.6; Abnova, Heidelberg, Germany), a polyclonal goat antibody against MDA5 (2.5 μg/ml; Imgenex, San Diego, CA), and a monoclonal mouse IgG1 antibody against RIG-I (2.5 μg/ml, clone Alme-1; Axxora, San Diego, CA).

Culture and stimulation of primary TECs

Primary TECs were isolated and cultured according to the method described previously [67] and stimulated with IFN-α2a and IFN-β1a (100–1000 U/ml; PBL InterferonSource, Piscataway, NJ), polyinosinic-polycytidylic acid (poly(I:C), 0.01–10 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands), 3pRNA (10–1000 ng/ml), a kind gift from Professor G Hartmann and Dr M Schlee (University Hospital Bonn, Germany), combined with Fugene HD (Roche, Mannheim, Germany) and/ or monoclonal IgM antibody (CH11) against CD95 (2 μg/ml; Beckman Coulter, Woerden, The Netherlands). The inhibitors used were chloroquine (10–100 μmol/l; Sigma-Aldrich), Z-IETD-FMK (20 μmol/l; Sigma-Aldrich), Q-VD-OPH (20 μmol/l; R&D systems, Minneapolis, MN), and compound A, kindly provided by Tetralogic Pharmaceuticals (Malvern, PA). Influenza A (strain A/PR/8/34) was a kind gift from Dr G Rimmelzwaan (Erasmus Medical Center Rotterdam, The Netherlands).

ELISA

The human TNF-α ELISA kit (Sanquin, Amsterdam, The Netherlands) and human IFN-β ELISA kit (Alexis Biochemicals, San Diego, CA) were used according to the instructions of the manufacturer.

Flow cytometry

TECs were stained with Annexin V (FITC conjugated; IQ products, Groningen, The Netherlands), propidium iodide (Sigma-Aldrich), FITC-labeled monoclonal IgG1 antibody against CD95 and isotype control (BD Biosciences, San Jose, CA), or 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3); Invitrogen, Breda, The Netherlands) and analyzed on a flow cytometer (FACSCalibur; BD Biosciences).

Western blot analysis

Cells were lysed in 1% Triton-X supplemented with protease inhibitor cocktail (Roche). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Supplementary Table S3 online depicts the primary antibodies used. Immunoreactive proteins were visualized with the Odyssey Infrared Imaging System (LI-COR Biotechnology, Bad Homburg, Germany).

Multiplex ligation–dependent probe amplification

The SALSA MLPA kit R011 Apoptosis (MRC-Holland, Amsterdam, The Netherlands) was used according to the manufacturer's protocol. Relative gene expression was defined as the ratio of each measured peak area to the combined peak area of all genes. The fold change (2-log) compared with unstimulated TECs was calculated and depicted as heat-map using the MultiExperiment Viewer software (version v4.6.1; Dana-Farber Cancer Institute, Boston, MA).

Statistical analysis

Statistical analyses were performed in Graph Pad Prism (version 5; GraphPad software, La Jolla, CA). dsRNA receptor transcript levels in biopsies were compared with Mann–Whitney *U*-tests. Individual values, corresponding to measurements in TEC cultures derived from different patients, were compared with paired Student's *t*-tests.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. TLR3 and RIG-I are expressed in renal tubuli during infection with CMV and EBV.
Figure S2. dsRNA receptor activation enhances the expression of pro-inflammatory mediators in TECs.
Figure S3. The TLR3 ligand poly(I:C) sensitizes TECs to CD95-mediated apoptosis.
Figure S4. dsRNA and CD95-mediated apoptosis is NFκB independent, caspase mediated and inhibited by IAPs.
Table S1. Patient characteristics.
Table S2. Primers used for PCR.
Table S3. Antibodies used for Western blot analysis.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Weikert BC, Blumberg EA. Viral infection after renal transplantation: surveillance and management. *Clin J Am Soc Nephrol* 2008; **3**(Suppl 2): S76–S86.
2. Staras SA, Dollard SC, Radford KW *et al.* Seroprevalence of cytomegalovirus infection in the United States, 1988–1994. *Clin Infect Dis* 2006; **43**: 1143–1151.
3. Egli A, Infanti L, Dumoulin A *et al.* Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis* 2009; **199**: 837–846.
4. Morris MC, Edmunds WJ, Hesketh LM *et al.* Sero-epidemiological patterns of Epstein-Barr and herpes simplex (HSV-1 and HSV-2) viruses in England and Wales. *J Med Virol* 2002; **67**: 522–527.
5. Boothpur R, Brennan DC. Human polyoma viruses and disease with emphasis on clinical BK and JC. *J Clin Virol* 2010; **47**: 306–312.
6. Becker JL, Miller F, Nuovo GJ *et al.* Epstein-Barr virus infection of renal proximal tubule cells: possible role in chronic interstitial nephritis. *J Clin Invest* 1999; **104**: 1673–1681.
7. Low J, Humes HD, Szczypka M *et al.* BKV and SV40 infection of human kidney tubular epithelial cells *in vitro*. *Virology* 2004; **323**: 182–188.
8. Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. *Nat Rev Immunol* 2008; **8**: 911–922.
9. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805–820.
10. Kawai T, Takahashi K, Sato S *et al.* IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005; **6**: 981–988.
11. Borden EC, Sen GC, Uze G *et al.* Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* 2007; **6**: 975–990.
12. Trinchieri G. Type I interferon: friend or foe? *J Exp Med* 2010; **207**: 2053–2063.
13. Besch R, Poeck H, Hohenauer T *et al.* Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells. *J Clin Invest* 2009; **119**: 2399–2411.
14. Lallemand C, Blanchard B, Palmieri M *et al.* Single-stranded RNA viruses inactivate the transcriptional activity of p53 but induce NOXA-dependent apoptosis via post-translational modifications of IRF-1, IRF-3 and CREB. *Oncogene* 2007; **26**: 328–338.
15. Chattopadhyay S, Marques JT, Yamashita M *et al.* Viral apoptosis is induced by IRF-3-mediated activation of Bax. *EMBO J* 2010; **29**: 1762–1773.
16. Paone A, Starace D, Galli R *et al.* Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-alpha-dependent mechanism. *Carcinogenesis* 2008; **29**: 1334–1342.
17. Peng S, Geng J, Sun R *et al.* Polyinosinic-polycytidylic acid liposome induces human hepatoma cells apoptosis which correlates to the up-regulation of RIG-I like receptors. *Cancer Sci* 2009; **100**: 529–536.
18. Weber A, Kirejczyk Z, Besch R *et al.* Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells. *Cell Death Differ* 2010; **17**: 942–951.
19. Galluzzi L, Brenner C, Morselli E *et al.* Viral control of mitochondrial apoptosis. *PLoS Pathog* 2008; **4**: e1000018.
20. Chavez-Galan L, renas-Del Angel MC, Zenteno E *et al.* Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol* 2009; **6**: 15–25.
21. Heutinck KM, ten Berge IJM, Hack CE *et al.* Serine proteases of the human immune system in health and disease. *Mol Immunol* 2010; **47**: 1943–1955.
22. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol* 2009; **10**: 348–355.
23. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; **87**: 99–163.
24. Heutinck KM, Kassies K, Florquin S *et al.* SerpinB9 expression in human renal tubular epithelial cells is induced by triggering of the viral dsRNA sensors TLR3, MDA5 and RIG-I. *Nephrol Dial Transplant*; e-pub ahead of print 13 December 2011.
25. Imaizumi T, Tanaka H, Matsumiya T *et al.* Retinoic acid-inducible gene-I is induced by double-stranded RNA and regulates the expression of CC chemokine ligand (CCL) 5 in human mesangial cells. *Nephrol Dial Transplant* 2010; **25**: 3534–3539.
26. Kariko K, Ni H, Capodici J *et al.* mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem* 2004; **279**: 12542–12550.
27. Tormo D, Checinska A, onso-Curbelo D *et al.* Targeted activation of innate immunity for therapeutic induction of autophagy and apoptosis in melanoma cells. *Cancer Cell* 2009; **16**: 103–114.
28. Le Goffic R, Pothlichet J, Vitour D *et al.* Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* 2007; **178**: 3368–3372.
29. Iwai A, Shiozaki T, Kawai T *et al.* Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1. *J Biol Chem* 2010; **285**: 32064–32074.
30. Ehrhardt C, Seyer R, Hrincius ER *et al.* Interplay between influenza A virus and the innate immune signaling. *Microbes Infect* 2010; **12**: 81–87.
31. Boonstra JG, van der Woude FJ, Wever PC *et al.* Expression and function of Fas (CD95) on human renal tubular epithelial cells. *J Am Soc Nephrol* 1997; **8**: 1517–1524.
32. Khan S, Koepke A, Jarad G *et al.* Apoptosis and JNK activation are differentially regulated by Fas expression level in renal tubular epithelial cells. *Kidney Int* 2001; **60**: 65–76.
33. Sun Y, Leaman DW. Involvement of Noxa in cellular apoptotic responses to interferon, double-stranded RNA, and virus infection. *J Biol Chem* 2005; **280**: 15561–15568.
34. Salaun B, Lebecque S, Matikainen S *et al.* Toll-like receptor 3 expressed by melanoma cells as a target for therapy? *Clin Cancer Res* 2007; **13**(15 Pt 1): 4565–4574.
35. Eldering E, Spek CA, Aberson HL *et al.* Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways. *Nucleic Acids Res* 2003; **31**: e153.
36. Srinivasula SM, Ashwell JD. IAPs: what's in a name? *Mol Cell* 2008; **30**: 123–135.
37. Vince JE, Wong WW, Khan N *et al.* IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007; **131**: 682–693.
38. Yew KH, Carsten B, Harrison C. Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/-9 in monocytic THP-1 cells. *Mol Immunol* 2010; **47**: 883–893.
39. Mezger M, Bonin M, Kessler T *et al.* Toll-like receptor 3 has no critical role during early immune response of human monocyte-derived dendritic cells after infection with the human cytomegalovirus strain TB40E. *Viral Immunol* 2009; **22**: 343–351.
40. Iwakiri D, Zhou L, Samanta M *et al.* Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from toll-like receptor 3. *J Exp Med* 2009; **206**: 2091–2099.

41. Samanta M, Iwakiri D, Kanda T *et al.* EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. *EMBO J* 2006; **25**: 4207–4214.
42. Tanner J, Weis J, Fearon D *et al.* Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 1987; **50**: 203–213.
43. Revello MG, Gerna G. Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev Med Virol* 2010; **20**: 136–155.
44. Sinclair J. Human cytomegalovirus: latency and reactivation in the myeloid lineage. *J Clin Virol* 2008; **41**: 180–185.
45. Nickeleit V, Mihatsch MJ. Polyomavirus nephropathy in native kidneys and renal allografts: an update on an escalating threat. *Transpl Int* 2006; **19**: 960–973.
46. Tsai JD, Lee HC, Lin CC *et al.* Epstein-Barr virus-associated acute renal failure: diagnosis, treatment, and follow-up. *Pediatr Nephrol* 2003; **18**: 667–674.
47. Hägele H, Allam R, Pawar RD *et al.* Double-stranded RNA activates type I interferon secretion in glomerular endothelial cells via retinoic acid-inducible gene (RIG)-1. *Nephrol Dial Transplant* 2009; **24**: 3312–3318.
48. Imaizumi T, Hatakeyama M, Yamashita K *et al.* Interferon-gamma induces retinoic acid-inducible gene-I in endothelial cells. *Endothelium* 2004; **11**: 169–173.
49. Imaizumi T, Hatakeyama M, Yamashita K *et al.* Double-stranded RNA induces the synthesis of retinoic acid-inducible gene-I in vascular endothelial cells. *Endothelium* 2005; **12**: 133–137.
50. van de Berg PJ, Heutinck KM, Raabe R *et al.* Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature. *J Infect Dis* 2010; **202**: 690–699.
51. Ribeiro A, Wornle M, Motamedi N *et al.* Activation of innate immune defense mechanisms contributes to polyomavirus BK-associated nephropathy. *Kidney Int* 2011; **81**: 100–111.
52. Mannon RB, Hoffmann SC, Kampen RL *et al.* Molecular evaluation of BK polyomavirus nephropathy. *Am J Transplant* 2005; **5**: 2883–2893.
53. Dessing MC, Bemelman FJ, Claessen N *et al.* Intragraft Toll-like receptor profiling in acute renal allograft rejection. *Nephrol Dial Transplant* 2010; **25**: 4087–4092.
54. Abend JR, Low JA, Imperiale MJ. Global effects of BKV infection on gene expression in human primary kidney epithelial cells. *Virology* 2010; **397**: 73–79.
55. Grinde B, Gayorfar M, Rinaldo CH. Impact of a polyomavirus (BKV) infection on mRNA expression in human endothelial cells. *Virus Res* 2007; **123**: 86–94.
56. Goubau D, Romieu-Mourez R, Solis M *et al.* Transcriptional re-programming of primary macrophages reveals distinct apoptotic and anti-tumoral functions of IRF-3 and IRF-7. *Eur J Immunol* 2009; **39**: 527–540.
57. Flur K, Allam R, Zecher D *et al.* Viral RNA induces type I interferon-dependent cytokine release and cell death in mesangial cells via melanoma-differentiation-associated gene-5: implications for viral infection-associated glomerulonephritis. *Am J Pathol* 2009; **175**: 2014–2022.
58. Chiron D, Pellat-Deceunynck C, Amiot M *et al.* TLR3 ligand induces NF-\{kappa\}B activation and various fates of multiple myeloma cells depending on IFN-\{alpha\} production. *J Immunol* 2009; **182**: 4471–4478.
59. Brenner D, Mak TW. Mitochondrial cell death effectors. *Curr Opin Cell Biol* 2009; **21**: 871–877.
60. Lopez H, Zhang L, George NM *et al.* Perturbation of the Bcl-2 network and an induced Noxa/Bcl-xL interaction trigger mitochondrial dysfunction after DNA damage. *J Biol Chem* 2010; **285**: 15016–15026.
61. Hagenbuchner J, Ausserlechner MJ, Porto V *et al.* The anti-apoptotic protein BCL2L1/Bcl-xL is neutralized by pro-apoptotic PMAIP1/Noxa in neuroblastoma, thereby determining bortezomib sensitivity independent of prosurvival MCL1 expression. *J Biol Chem* 2010; **285**: 6904–6912.
62. Schutze S, Tchikov V, Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. *Nat Rev Mol Cell Biol* 2008; **9**: 655–662.
63. Jost PJ, Grabow S, Gray D *et al.* XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature* 2009; **460**: 1035–1039.
64. Du C, Jiang J, Guan Q *et al.* Renal tubular epithelial cell self-injury through Fas/Fas ligand interaction promotes renal allograft injury. *Am J Transplant* 2004; **4**: 1583–1594.
65. Lorz C, Ortiz A, Justo P *et al.* Proapoptotic Fas ligand is expressed by normal kidney tubular epithelium and injured glomeruli. *J Am Soc Nephrol* 2000; **11**: 1266–1277.
66. Lorz C, ito-Martin A, Boucherot A *et al.* The death ligand TRAIL in diabetic nephropathy. *J Am Soc Nephrol* 2008; **19**: 904–914.
67. van Kooten C, Lam S, Daha MR. Isolation, culture, characterization and use of human renal tubular epithelial cells. *J Nephrol* 2001; **14**: 204–210.