Cyclosporine A Induces MicroRNAs Controlling Innate Immunity during Renal Bacterial Infection

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Keywords

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Abstract

Urinary tract infections (UTIs) mainly due to uropathogenic *Escherichia coli* (UPEC) are one of the most frequent complications in kidney-transplanted patients, causing significant morbidity. However, the mechanisms underlying UTI in renal grafts remain poorly understood. Here, we analysed the effects of the potent immunosuppressive agent cyclosporine A (CsA) on the activation of collecting duct cells that represent a preferential site of adhesion and translocation for UPEC. CsA induced the inhibition of lipopolysaccharide-induced activation of collecting duct cells due to the down-regulation of the expression of TLR4 via the microRNA Let-7i. Using an experimental model of ascending UTI, we showed that the pretreatment of mice with CsA prior to infection induced a marked fall in cytokine production by collecting duct cells, neutrophil recruitment, and a dramatic rise of bacterial load, but not in infected TLR4-defective mice kidneys. This effect was also observed in CsA-treated infected kidneys, where the expression of Let-7i was increased. Treatment with a synthetic Let-7i mimic reproduced the effects of

CsA. Conversely, pretreatment with an anti-Let-7i antagonised the effects of CsA and rescued the innate immune response of collecting duct cells against UPEC. Thus, the utilisation of an anti-Let-7i during kidney transplantation may protect CsA-treated patients from ascending bacterial infection.

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Introduction

Despite the improvement of surgical procedures and immunosuppressive therapies, urinary tract infections (UTIs) still represent the most frequent bacterial infections in kidney-transplanted recipients, and are responsible of morbidity and renal graft dysfunction [1–3]. Uropathogenic *Escherichia coli* (UPEC) is the most common cause of UTIs in the general population as well as in kidney-transplanted patients [1, 4–6].

UPEC may ascend along the urinary tract and colonise the kidneys [7]. Members of the family of Toll-like receptors (TLRs), particularly TLR4, which senses lipopolysaccharide (LPS) from gram-negative bacteria, expressed in renal parenchymal cells and bone marrow-derived cells, play key roles in the initiation of the innate response for

the defense of the host and efficient renal bacterial clearance [8–10]. Using experimental models of ascending UTIs, we have shown that UPEC preferentially adheres to the parenchymal medullary collecting duct (MCD), which in turn develops TLR4-dependent and independent signaling pathways leading to the production of cytokines and the recruitment of polymorphonuclear neutrophils (PMNs) to the site of inflammation [11, 12].

Long-term immunosuppressive therapy in the context of transplantation increases the susceptibility to infection. However, the mechanisms by which immunosuppressive agents may favour renal bacterial infection still remain poorly understood. The calcineurin inhibitor cyclosporine A (CsA) is one of the most potent immunosuppressive agents used in the prevention of renal graft rejection. However, CsA can be nephrotoxic and causes chronic interstitial fibrosis [1, 2, 13]. High concentrations of CsA trigger the apoptosis of renal tubule cells [14]. Interestingly, CsA was also shown to impair cytokine-induced matrix-metalloproteinase-9 associated with an inhibition of the IL-1 β -induced activation of the transcription factor NF- κ B and mitogen-activated protein (MAP) kinase c-Jun N-terminal kinase (JNK) in rat mesangial cells [15]. Moreover, we previously showed that CsA impairs nucleotide binding oligomerisation domain (Nod1)-mediated innate antibacterial renal defenses in mice and human transplant recipients [16, 17]. These findings raised the possibility that CsA can impair the activation of MCD cells caused by LPS and UPEC, and thereby favour the intrarenal colonisation of UPEC.

Recent advances in genomics, sequencing technologies, and bioinformatics have generated an explosion of genetic information, highlighting the importance of understanding mechanisms involved in gene expression and regulation. Gene expression can be regulated by different mechanisms, such as through transcription factors, alternative splicing, or epigenetics and gene silencing by small RNAs, including microRNAs (miRs). miRs are highly conserved non-coding small single-stranded RNA molecules that have emerged as master regulators of gene expression in plants and animals, from nematodes to arthropods and humans, and even viruses. miRs have been shown to be major actors in the tuning of a broad range of developmental, cell differentiation, and cell maintenance functions. miRs have been especially implicated in the regulation of inflammatory processes and some of them, such as miR-146a, miR-155, the miR-17-92 cluster, miR-21, miR-125b, miR-203, or the let-7 family, have been shown to regulate innate immune signaling [18–21]. However, the association between miR dysregulation and

the pathogenesis of inflammatory kidney diseases is poorly defined.

We therefore analysed the effects of CsA on TLR4-mediated cell activation in immortalised renal collecting duct cells, mpkCCD_{cl4}, and in primary cultures of MCD dissected from the kidneys of wild-type C3H/HeN (*Lps*ⁿ) mice and *Trl4*-inactivated C3H/HeJ (*Lps*^d) mice. The involvement of miRs was also assessed and the consequence of long-term administration of CsA on the renal innate response and renal bacterial burden were also analysed. The present findings establish that CsA alters *Trl4* gene expression in an miR-dependent manner and impairs TLR4-mediated cell activation in MCD cells, favouring bacterial infections.

Materials and Methods

Cell Culture and Incubations

mpkCCD_{cl4} cells and RAW 264.7 cells were cultured as described [11, 22, 23]. *Escherichia coli* K12 D31m4 LPS (List Biological Laboratories) was used for the stimulations. The purity of the LPS preparation was confirmed by the complete absence of stimulating activity on peritoneal macrophages isolated from *Trl4*-deficient mice. The absence of endotoxin in the CsA solution was checked with the chromogenic QCL-1000 limulus amoebocyte lysate assay (Lonza) as described previously [24]. CsA was preincubated for 18 h prior to LPS stimulation. For miRNA silencing, an miR inhibitor against Dicer and Drosha was used together with a Cy3-labelled negative control miR inhibitor (Ambion) at a final concentration of 100 nM as previously described [24]. The same protocol was used for the transfection of anti-miR (anti-Let-7i inhibitor; Ambion) and mature microRNA mimics (mmu-Let-7i miRIDIAN mimic, Dharmacon RNAi Technologies).

Bacteria

We used the HT7 UPEC strain isolated from the urine of a woman with acute pyelonephritis. HT7 expresses *papGII* encoding the PagG subunit (allelic variant II) of P fimbriae frequently expressed in pyelonephritic *E. coli*, but lacks the virulent factors *cnf1*, *hly*, and *sat* encoding necrotising and vacuolating cytotoxins.

miRNA PCR Array

mpkCCD_{cl4} cells were or were not incubated with CsA, and miRs were isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Samples were then sent to Qiagen for the performance of a miScript miRNA PCR array.

Ethics Statement

All animal experiments were approved by and conducted in accordance with the guidelines of the French Agricultural Office and in compliance with the French and European regulations on Animal Welfare (Service de la Protection et Santé Animale; Approval No. 75-687, revised 2008) and with Public Health Service recommendations. All efforts were made to minimise the suffering of the mice.

Microdissection and Culture of MCDs

Experiments were also carried out on primary cultures of isolated MCDs dissected from the kidneys of 9- to 13-week-old C3H/HeJ *Lps^d* mice, C3H/HeOuJ *Lpsⁿ* mice, or wild-type C57BL/6 mice as described previously [11]. Pools of isolated MCD (8–12 fragments) were seeded onto collagen-coated, 1- μ m-pore-sized Transwell permeable filters and grown to confluence as described already [25]. Experiments were carried out 2 weeks after seeding, using confluent cells with a transepithelial electrical resistance of $\geq 4,000 \Omega \cdot \text{cm}^2$. All experiments were carried out in accordance with French legislation governing animal studies.

Renal Retrograde Infection in Mice

Adult female wild-type mice (8–10 weeks old, supplied by the Centre d'Élevage Janvier, Le Genest-Saint-Isle, France), from the C3H/HeJ *Lps^d*, C3H/HeOuJ *Lpsⁿ*, or C57BL/6 genetic background were used. The mice were infected with the UPEC strain HT7 (10^8 bacteria in 50 mL OF sterile PBS) introduced via the transurethral route into the bladder as PREVIOUSLY described [11]. A total of 100 mL of CsA (5 mg/kg; Neoral, Novartis), or its vehicle (castor oil) were administered subcutaneously to mice for 5 days before the inoculation of UPEC. Injections of anti-miR (anti-Let-7i inhibitor, Ambion) or mature miR mimics (mmu-let-7i miRIDIAN mimic, Dharmacon RNAi Technologies) were performed once before the inoculation of bacteria using in vivo-jetPEI® (Polyplus transfection) according to the manufacturer's instructions. Bacterial loads (CFU) in the kidneys were determined 24 h after infection by plating.

Histological and Immunohistochemical Studies

Kidneys were fixed in Dubosq-Brazil solution, rinsed in PBS, embedded in paraffin, and stained with haematoxylin-eosin. The degree of inflammatory changes was assessed using a semiquantitative graded scale: 0, no abnormality; 1, mild lesions affecting <25% of kidney samples; 2, moderate lesions affecting 25–50% of kidney samples; 3, diffuse lesions affecting 75% of kidney samples.

Real-Time PCR and Reverse Transcription PCR

Total RNA from mouse kidneys, mpkCCD_{cl4}, MCD, RAW264.7, and BMM was purified with RNA NOW (Ozyme) and reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (200 U/ μ L, Invitrogen) for 60 min at 42°C in the presence of dNTP and oligo dT. The mouse Tlr4, Mip-2, Let-7i, siRNA Dicer, siDrosha, GAPDH, and Sno (PrimePCR™ SYBR® Green Assay, Bio-Rad) cDNA was subjected to quantitative real-time PCR using the SYBR® Green method. Analyses were performed with a CFX96 real-time PCR detection system (Bio-Rad). Each sample was amplified in duplicate, and normalised versus the endogenous control. Results were calculated using the $2^{-\Delta\Delta C_T}$ method, and presented as the fold induction of the target gene transcript under stimulated versus unstimulated conditions.

Immunoblot and Endotoxin Quantification

Immunoblotting was performed as recently described [11]. Antibodies against TLR4 (rabbit polyclonal, Santa Cruz Biotechnology), total and phosphorylated ERK, JNK, NF- κ B, p38 (mouse monoclonal, Cell Signaling Technology), and β -actin (N-terminal domain; Sigma-Aldrich, Taufkirchen, Germany) were used in combination with peroxidase-labelled goat-anti-mouse or goat-anti-rabbit secondary antibodies (Jackson ImmunoResearch).

ELISA

Cxcl1 and Cxcl2 secretion in cell supernatants was then determined using ELISA kits (R&D Systems) according to the manufacturer's instructions. For in vivo Cxcl1 and Cxcl2 measurements, kidneys from *Lpsⁿ*, *Lps^d*, and wild-type mice infected with the UPEC strain HT7 isolates were homogenised in 1 mL of PBS and kept at -80°C until used. Tissue samples were then thawed and assayed to measure the levels of Cxcl1 and Cxcl2 production. Results were standardised to the amount of protein detected for each sample using the Bio-Rad protein assay with BSA as the standard. Myeloperoxidase (MPO) activity was measured using the HyCult Biotechnology ELISA kit.

Statistical Analysis

Results are expressed as means $\pm$ SE, and are representative of at least 3 independent experiments for each of the experimental conditions tested. Differences were analysed with the unpaired Student *t* test compared to the control. $p < 0.05$ was considered significant.

Results

CsA Selectively Impairs TLR4 Expression and LPS-Induced Cellular Activation in Renal Epithelial Collecting Duct Cells

Renal MCD cells are the first cells in contact with UPEC during ascendant bacterial infection and initiate the inflammatory response mainly in a TLR4-dependent signaling pathway [11]. To check whether CsA has a specific effect on epithelial cells, we performed experiments on a highly differentiated cell line of MDC cells (mpkCCD_{cl4}), as well as primary cultures of MCD cells microdissected from the kidneys of wild-type mice, versus bone marrow-derived immune cells (macrophage-like RAW 264.7 cell line and freshly isolated bone marrow macrophages; BMMs). Confluent mpkCCD_{cl4} cells, RAW 264.7 cells, primary cultures of MCD, and BMMs

Fig. 1. CsA decreases cellular activation in renal collecting duct cells. **a** Variation in the levels of *Tlr4* mRNAs measured by quantitative real-time PCR in confluent cultures of mpkCCD_{cl4}, MCD, RAW 264.7, and BMM cells incubated with various concentrations of CsA for 18 h compared to untreated controls. **b** Immunoblot analysis of TLR4 proteins and the corresponding β -actin in mpkCCD_{cl4} cells incubated with or without various concentrations of CsA for 18 h. **c** Representative immunoblot showing the effect of CsA at 10^{-8} M on the phosphorylation (p) of p65, ERK, and JNK proteins from LPS-treated mpkCCD_{cl4} cells. **d** CXCL1 (upper line) and CXCL2 (lower line) production measured by ELISA in wild-type mpkCCD_{cl4}, primary cultured MCD, RAW 264.7, and BMM cells incubated with various concentrations of CsA for 18 h, then with or without 10 ng/mL of LPS. Values are presented as the mean $\pm$ SE compared to the control. * $p < 0.05$, *** $p < 0.001$.

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Fig. 2. CsA induces miR, modulating TLR4 expression, and LPS-induced cellular activation. **a** Relative expression of *Tlr4* mRNA in mpkCCD_{cl4} cells transfected or not with a siRNA against Drosha or Dicer, then treated or not with CsA (10^{-8} M) for 18 h. **b** CXCL1 and CXCL2 production measured by ELISA in the supernatant of mpkCCD_{cl4} cells transfected or not with a siRNA against Drosha

or Dicer, then treated or not with CsA and stimulated with LPS for 6 h. **c** Quantitative RT-PCR expression analysis of miRNA Let-7i in mpkCCD_{cl4} cells treated (CsA) or not (Co) with CsA (10^{-8} M for 18 h). Values are presented as the mean $\pm$ SE compared to the control. *** $p < 0.001$.

were incubated with different concentrations of CsA for 18 h at 37°C. We carefully checked that these concentrations did not affect cell viability with an MTT assay 24 h after incubation with CsA (online suppl. Fig. 1a; see www.karger.com/doi/10.1159/000480248 for all online suppl. material). We then analysed the expression of *Tlr4* mRNA and the results showed that CsA (10^{-10} to 10^{-7} M) significantly decreased the expression of *Tlr4* mRNA in a dose-dependent manner in mpkCCD_{cl4} cells and primary cultures of MCD cells (Fig. 1a), with a 50% inhibition for 10^{-8} M CsA. However, CsA had no effect on *Tlr4* mRNA expression, neither in RAW 264.7 cells nor in BMs (Fig. 1a). We then tested whether the downregulation of *Tlr4* mRNA in renal epithelial cells caused by CsA was specific by checking the expression of other *Tlrs* under the same experimental conditions. Our results

showed no effect of CsA on the expression of *Tlr2*, *Tlr3*, *Tlr5*, and *Tlr9* in mpkCCD_{cl4} cells, as well as on the expression of *Tlr2*, *Tlr3*, and *Tlr9* in RAW 264.7 cells (data not shown). TLR-mediated recognition of bacterial components induces a cascade of signaling events that finally leads to the activation of the transcriptional factor NF- κ B and MAP kinases, which are critical in the regulation of genes involved in inflammation [26]. Western blot analyses were performed to check the TLR4 expression and the results showed that CsA induces a downregulation of TLR4 protein level in a dose-dependent manner, specifically in renal epithelial mpkCCD_{cl4} cells (Fig. 1b). To identify the influence of CsA on LPS-induced cell activation, mpkCCD_{cl4} cells were then incubated without or with 10 ng/mL of LPS for 6 h after incubation with CsA (10^{-8} M) for 18 h. LPS induced the phosphorylation of the

Fig. 3. CsA induces *Let-7i* expression targeting TLR4 in renal epithelial cells. **a** Quantitative RT-PCR expression analysis of *Let-7i* levels in mpkCCD_{cl4} and RAW264.7 cells treated with CsA, tacrolimus (Tacro), sirolimus (Siro), and 11R-Vivit (Vivit) for 18 h (10⁻⁸ M). **b** *Let-7i* expression in mpkCCD_{cl4} cells transfected with a *Let-7i* mimic or a mimic control (mimic Co). **c** *Tlr4* mRNA relative expression and CXCL1 (grey bars) and CXCL2 (white bars) secretion measured by ELISA in mpkCCD_{cl4} cells transfected or not with a *Let-7i* mimic or mimic control, then treated or not with CsA for

18 h, and then stimulated (right panel) or not (left panel) with LPS for 6 h. **d** *Let-7i* expression in mpkCCD_{cl4} cells transfected with an anti-*Let-7i* or an anti-miR control (anti-Co) after stimulation with CsA. **e** *Tlr4* mRNA relative expression and CXCL1 (grey bars) and CXCL2 (white bars) secretion measured by ELISA in mpkCCD_{cl4} cells transfected or not with an anti-*Let-7i* or anti-miRNA control (anti-Co), then treated or not with CsA for 18 h, and stimulated (right panel) or not (left panel) with LPS for 6 h. Values are presented as the mean $\pm$ SE compared to the control. *** $p < 0.001$.

NF- κ B subunit p65 as well as of the MAP kinases extracellular signal-regulated kinase (ERK)1/2 and JNK (Fig. 1c). However, with the preincubation with CsA, their activation was impaired (Fig. 1c; online suppl. Fig. 1b). We then analysed the LPS-induced secretion of the chemokine Cxcl-2 by ELISA. We checked that Cxcl-2 secretion was TLR4 dependant in mpkCCD_{cl4} cells (online suppl. Fig. 1c), in MCD cells (online suppl. Fig. 1d), in RAW 264.7 cells (online suppl. Fig. 1e), as well as in BMMs (online suppl. Fig. 1f) by using siRNA targeting TLR4 on LPS-treated cells. Cxcl-2 has been shown to be secreted by renal epithelial cells after TLR4 activation by UPEC and transcriptionally regulated by NF- κ B [11, 27]. The results showed that LPS induces Cxcl2 secretion in mpkCCD_{cl4} cells, RAW 264.7 cells, primary cultures of MCD cells, as well as in BMMs. Preincubation with CsA induces a decrease of Cxcl2 secretion only in mpkCCD_{cl4} cells and in primary cultures of MCD cells in a dose-dependent manner. These results indicate that CsA inhibits LPS-induced cell activation specifically in MCD cells by decreasing the *Tlr4* mRNA expression.

CsA Decreases Tlr4 Expression through a miR-Dependent Process in Renal Collecting Duct Cells

miRs can modulate the expression of mRNAs and therefore decrease the expression of key proteins involved in major signaling pathways [18–21]. To check whether the downregulation of *Tlr4* expression in collecting duct cells by CsA is induced by the presence of miRs, we depleted the whole miRs from the cells before incubation with LPS. In the canonical pathway of miRs biogenesis, the transcript (primary precursor, pri-miR) forms a microprocessor complex with the RNase III enzyme Drosha and the dsRNA-binding protein DGCR8/Pasha in the nucleus [28]. The pri-miR was then cut in an approximate 70-nucleotide precursor hairpin (pre-miR), which is translocated to the cytoplasm by Exportin-5. In the cytoplasm, pre-miRs were processed to an approximate 21-nucleotide miR/miR duplex by a complex formed by the RNase III enzyme Dicer and TRBP/PACT/Loqs. One strand of the duplex was loaded onto Argonaute proteins and incorporated into the miR-induced silencing complex, miRISC, whereas the other strand was released and degraded. To transiently deplete mature miRs from the mpkCCD_{cl4} cells, we transfected them with a silencing RNA against Drosha or Dicer before the incubation with CsA, and then measured the *Tlr4* mRNA expression by quantitative PCR. The results showed that the CsA-induced decrease of *Tlr4* mRNA expression was antagonised when silencing either Dro-

sha or Dicer, the 2 major enzymes allowing miR maturation (Fig. 2a). This result suggests that the downregulation of the expression of *Tlr4* mRNA caused by CsA occurs through a mechanism involving miRs. We then stimulated the cells with LPS after the silencing of Drosha or Dicer and the preincubation with CsA to check the levels of Cxcl1 and Cxcl2 secretion (Fig. 2b). The results showed that cytokine secretion was restored when the cells were depleted of mature miRs, showing that the absence of mature miRs allows the activation of the collecting duct cells by LPS in the presence of CsA. To identify which miR could be involved in this regulation, we performed an miR PCR array assay on mpkCCD_{cl4} cells incubated or not with CsA (Fig. 2c). After the data analysis, we could identify miRs specifically modulated in the presence of CsA. Among them, Let-7i could be possibly involved in the regulation of the *Tlr4* gene. Therefore, mpkCCD_{cl4} cells and RAW 264.7 cells were incubated with CsA and checked for the expression of Let-7i. The results showed that Let-7i was induced in renal collecting duct cells, but not in macrophages, in the presence of CsA (Fig. 2d). Overall, these results suggest that CsA induces Let-7i specifically in renal collecting duct cells, and that this miR might be responsible for the downregulation of *Tlr4* mRNA expression and the subsequent inhibition of LPS-induced cell activation.

CsA Induces Let-7i Expression Targeting TLR4 in Renal Collecting Duct Cells

CsA forms a complex with cyclophilin, blocking the calcineurin-calmodulin-induced phosphorylation of nuclear factor of activated T cells (NFAT) transcription factor for IL-2 production. Similarly to CsA, tacrolimus (also known as FK506) is another macrolide antibiotic used as an immunosuppressive agent that interferes with

Fig. 4. CsA impairs the host defence during UTI by decreasing the expression of TLR4 in epithelial cells. **a** Bacterial load in kidneys of *Lpsⁿ* and *Lps^d* mice treated with CsA (5 mg/kg) or its vehicle for 5 days and 24 h after UPEC infection. **b** Illustrations of nodular infiltrates in fixed day-3 postinfected kidneys. Scale bars, 50 μ m. MPO activity (**c**) and CXCL1 and CXCL2 (**d**) secretion in the 24 h postinfected kidneys from vehicle-treated and CsA-treated *Lpsⁿ* or *Lps^d* mice. **e** *Let-7i*, *Tlr4*, *Cxcl1*, and *Cxcl2* mRNA expression analyses measured by quantitative real-time PCR in kidneys from vehicle-treated and CsA-treated *Lpsⁿ* or *Lps^d* mice after UPEC inoculation. **f** Relative expression of *Let-7i*, *Tlr4*, *Cxcl1*, and *Cxcl2* mRNAs measured by quantitative real-time PCR in confluent cultures of *Lpsⁿ* or *Lps^d* MCD cells incubated with vehicle or CsA for 18 h and compared to untreated MCD cells. Values are presented as the mean $\pm$ SE compared to the control. *** $p < 0.001$.

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the intracytoplasmic calcineurin system to inhibit NFAT [29]. On the other hand, sirolimus (also known as rapamycin) is a macrocyclic lactone also used as an immunosuppressive agent although it acts differently to the other 2. Even though it structurally resembles tacrolimus and binds to the same intracellular protein immunophilin, it blocks the activation of mTOR, a serine protein kinase important for cell cycle progression [30]. mpkCCD_{cl4} cells and RAW 264.7 cells were incubated with CsA, tacrolimus, sirolimus, and 11R-Vivit, a cell-permeable peptide that specifically inhibits the interaction of calcineurin-NFATs without affecting calcineurin phosphatase activity [31]. The results showed that CsA and tacrolimus induce Let-7i expression in mpkCCD_{cl4} cells but not in RAW 264.7 cells (Fig. 3a). However, incubation with sirolimus did not induce Let-7i expression, neither in mpkCCD_{cl4} cells nor in RAW 264.7 cells. Incubation with 11R-Vivit had the same effect as CsA and tacrolimus, inducing the expression of Let-7i specifically in mpkCCD_{cl4} cells (Fig. 1a). These results indicate that calcineurin inhibitors induce Let-7i expression in renal collecting duct cells. The Let-7 family, and especially Let-7i, has already been shown to interfere with TLR4 signaling in human biliary epithelial cells during *Cryptosporidium parvum* infection [32]. Functional studies in renal collecting duct cells were performed to link TLR4 and Let-7i in our infection model. mpkCCD_{cl4} cells were transfected with a synthetic Let-7i mimic or a mimic control and then incubated or not with CsA. The presence of Let-7i after Let-7i mimic transfection in mpkCCD_{cl4} cells was checked by quantitative PCR (Fig. 3b). In the presence of CsA alone, *Tlr4* expression was downregulated and the same effect could be observed in mpkCCD_{cl4} transfected with the Let-7i mimic in the absence of CsA (Fig. 3c, left panel). After LPS stimulation, Cxcl1 and Cxcl2 secretion were assessed by ELISA (Fig. 3b). In the presence of CsA and LPS, cytokine secretion was impaired in mpkCCD_{cl4} cells. In the same way, cytokine secretion was also impaired in the presence of LPS and in the absence of CsA in mpkCCD_{cl4} cells transfected with the Let-7i mimic. On the other hand, we used an anti-Let-7i mimic to inhibit the expression of Let-7i during CsA incubation. We checked the inhibition of the expression of Let-7i in the presence of CsA by quantitative PCR (Fig. 3d). *Tlr4* expression decreased in the presence of CsA, but was restored in the presence of anti-Let-7i and CsA (Fig. 3e, left panel). Similarly, in the presence of LPS, Cxcl1, and Cxcl2, secretion was inhibited in the presence of CsA and restored in the presence of an anti-Let-7i and CsA together (Fig. 3e, right panel).

Since we previously showed that CsA impairs Nod1 expression in renal tubular cells infected with UPEC [16], we checked that Let-7i had no effect on Nod1 expression (online suppl. Fig. 2). Altogether, these results suggest that CsA induces the miR Let-7i in renal collecting duct cell, which in turn downregulates the expression of *Tlr4* and downstream signaling.

CsA-Induced Inhibition of the TLR4-Mediated Innate Host Defense in Response to UPEC Leads to Exacerbated Kidney Infection in vivo

The results from the in vitro studies indicated that CsA acts as a potent inhibitor of TLR4-mediated innate immunity. However, the inhibitory mechanism(s) of the calcineurin inhibitor on the innate immune response are still debated. Recently, we have shown that CsA decreases renal susceptibility to UPEC through an NFATc1-dependent ignition of Nod1, which is involved in the recruitment of phagocytic cells to the site of inflammation [16, 17]. Because TLR4 has been shown to also play a key role in renal host defense against pyelonephritic-associated UPEC, the question arises as to whether CsA also impairs the TLR4-mediated immune response during UTI. To answer this question, adult female C3H/HeN *Lps*ⁿ mice and C3H/HeJ *Lps*^d mice (8 mice per group) were left untreated or received a daily subcutaneous injection of CsA (5 mg/kg) diluted in endotoxin-free castor oil for 5 days before the infection experiments. As controls, C3H/HeN *Lps*ⁿ mice received a subcutaneous injection of castor oil alone for 5 days. The mice were given an intraurethral injection of UPEC. Thereafter, bacterial loads in the kidney, histology, MPO activity, Cxcl1 and Cxcl2 expression and secretion, as well as TLR4 expres-

Fig. 5. Treatment with a synthetic Let-7i mimics the effects of CsA in kidneys colonised by UPEC. **a** Bacterial load in 24 h postinfected kidneys of wild-type mice treated with CsA (5 mg/kg) for 5 days or with a synthetic Let-7i mimic (mimic miRNA) intraperitoneally injected (2 nmol/mice) just before UPEC (HT7) infection. **b** Illustrations of nodular infiltrates in fixed day-3 postinfected kidneys from wild-type mice treated with CsA (5 mg/kg) for 5 days or with a mimic miRNA intraperitoneally injected (2 nmol/mice) just before the HT7 infection. Scale bars, 50 μ m. **c** MPO activity in the 24 h postinfected kidney homogenates from wild-type mice treated with CsA or mimic miRNA. **d** Production of CXCL1 and CXCL2 in the 24 h postinfected kidneys from mimic miRNA-treated or CsA-treated wild-type mice. **e** Relative expression of *Let-7i*, *Tlr4*, *Cxcl1*, and *Cxcl2* mRNAs measured by quantitative real-time PCR in kidneys from wild-type mice treated or not with the Let-7i mimic miRNA, and stimulated or not with CsA for 5 days before the infection by HT7. Values are presented as the mean $\pm$ SE compared to the control. *** $p < 0.001$.

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sion were analysed 24 h after the transurethral inoculation of the UPEC strain HT7 [33]. *Lps^d* mice were more susceptible to infection since the renal bacterial burden at 24 h after challenge was significantly greater than in kidneys from *Lpsⁿ* mice (Fig. 4a). In the presence of CsA, the bacterial load in the kidneys of *Lpsⁿ* mice was dramatically increased, but no effect was had on the bacterial load in the kidneys of *Lps^d* mice. The same tendency was observed in the bladder of the infected mice (online suppl. Fig. 3). These results suggest that CsA favours bacterial proliferation in the kidneys though a process involving TLR4. Infiltrating cells surrounding tubules, including MCDs, as well as damage in the kidneys were detected in *Lpsⁿ* mice and increased when they were treated with CsA, and also to a lower extent in *Lps^d* kidneys colonised with UPEC (Fig. 4b). Neutrophil migration was assessed by measuring MPO activity in renal homogenates. As previously reported, MPO activity was lower in *Lps^d* than in *Lpsⁿ* kidneys colonised by UPEC (Fig. 4c). CsA treatment caused a marked decrease in MPO activity in the kidneys of UPEC-infected *Lpsⁿ* mice as compared to non-CsA-treated infected *Lpsⁿ* mice (Fig. 4c). CsA treatment had no effect on MPO activity from the kidneys of UPEC-infected *Lps^d* mice. Chemoattractant chemokines, such as Cxcl1 (KC) and Cxcl2 (MIP-2), play a key role in the migration of PMNs to mucosal sites of inflammation to produce efficient bacterial clearance [11, 16]. Cxcl1 and Cxcl2 secretion was assessed by ELISA in the kidney. Consistent with these findings, the production of Cxcl1 and Cxcl2, the 2 main neutrophil chemoattractant chemokines, was significantly lower in the infected kidneys of *Lpsⁿ* mice pretreated with CsA than in those of untreated mice (Fig. 4d). The effects of CsA appeared to be related to the alteration of TLR4 signaling, since the Cxcl1 and Cxcl2 protein levels measured in the infected *Lps^d* kidneys, which were significantly lower than in those of infected *Lpsⁿ* kidneys, were not affected by the CsA treatment (Fig. 4d). The relative expression of *Let-7i* and *Tlr4* mRNA was assessed by quantitative PCR in the whole kidney (Fig. 4e, left and central panels) and in microdissected MCD cells isolated from kidneys after CsA treatment (Fig. 4f, left and central panels). Consistent with our in vitro data, whereas *Let-7i* expression was increased, *Tlr4* mRNA expression was inversely decreased in the whole kidney, and especially in MCD cells. The relative levels of *Cxcl1* and *Cxcl2* mRNA expression after CsA treatment and/or UPEC infection was also measured in the whole kidney (Fig. 4e, right panel) and in MCD cells microdissected after infection (Fig. 4f, right panel). Administration of

CsA for 5 days before infection induced a significant decrease in *Cxcl1* and *Cxcl2* mRNA expression in the whole kidney of *Lpsⁿ* mice, as well as in MCD cells. *Cxcl1* and *Cxcl2* mRNA expression was decreased in the whole kidney and the MCD of infected *Lps^d* mice compared to infected *Lpsⁿ* mice, but the treatment with CsA did not have any effect. These findings demonstrate that CsA influences inflammatory responses in vivo, and further suggest that the suppressor effect of CsA is mainly restricted to TLR4-dependent immune activation.

Administration of a Synthetic Let-7i Mimics the Deleterious Effects of CsA on Renal Bacterial Defense

In order to analyse the effect of in vivo administration of a synthetic *Let-7i* mimic, wild-type mice were left untreated or received a daily subcutaneous injection of CsA (5 mg/kg) diluted in endotoxin-free castor oil for 5 days before infection experiments. Before the infection with UPEC, a group of mice untreated with CsA received an intraperitoneal injection of 2 nmol of *Let-7i* mimic per mouse. We checked the presence of the *Let-7i* mimics by quantitative PCR and FACS on microdissected MCD cells from the tubules of the injected mice, as previously shown [34]. At 24 h after infection, the bacterial load in the kidneys of mice treated with CsA was increased compared to CsA-untreated mice (Fig. 5a). Interestingly, the bacterial load in the kidneys of infected mice treated with the *Let-7i* mimic exhibited an equivalent increase to the CsA-treated mice (Fig. 5a). The same results were observed in the bladder of infected mice (online suppl. Fig. 4). Similar results were also observed for kidney damage (Fig. 5b), MPO activity (Fig. 5c), and CXCL1 and CXCL2 secretion in the kidney (Fig. 5d). As a control, the level of *Let-7i* expression in the kidney of the

Fig. 6. Treatment with an anti-*Let-7i* antagonises the effects of CsA in kidneys colonised by UPEC. **a** Bacterial load in 24 h postinfected kidneys of wild-type mice treated or not with CsA (5 mg/kg) for 5 days or with an anti-*Let-7i* intraperitoneally injected (2 nmol/mice) just before the UPEC (HT7) infection. **b** Illustrations of nodular infiltrates in fixed day-3 postinfected kidneys from wild-type mice treated with CsA (5 mg/kg) for 5 days or with an anti-*Let-7i* intraperitoneally injected (2 nmol/mice) just before the HT7 infection. Scale bars, 50 μ m. **c** MPO activity in the 24 h postinfected kidney homogenates from wild-type mice with CsA or anti-*Let-7i* treatment. **d** Production of CXCL1 and CXCL2 in the 24 h postinfected kidneys from anti-*Let-7i*-treated and CsA-treated wild-type mice. **e** Relative expression of *Let-7i*, *Tlr4*, *Cxcl1*, and *Cxcl2* mRNAs measured by quantitative real-time PCR in kidneys from wild-type mice treated or not with an anti-*Let-7i*, and stimulated or not with CsA for 5 days before the infection by HT7. Values are presented as the mean $\pm$ SE compared to the control. ** $p < 0.01$, *** $p < 0.001$.

(For figure see next page.)

UPEC-infected mice pretreated with the mimic was checked (Fig. 5e, left panel). The consequences of the injection of the Let-7i mimic on *Tlr4* and *Cxcl1* and *Cxcl2* mRNA expression were then analysed. The results showed that Let-7i mimic considerably decreased the expression of *Tlr4*, *Cxcl1*, and *Cxcl2* to almost the same extent as CsA treatment does (Fig. 5e, central and right panels). Overall, the results showed that the injection of Let-7i mimics the effects of CsA in renal epithelial cells during UPEC infection by decreasing *Tlr4* expression, *Cxcl1* and *Cxcl2* expression and protein secretion, and MPO activity, and increases the UPEC bacterial load and kidney damage.

An Anti-Let-7i Antagonises the Inhibitory Action of CsA in Kidneys during UPEC Infection

The effects of an injection of an anti-Let-7i were analysed in mice treated with CsA. Wild-type mice were left untreated or received a daily subcutaneous injection of CsA (5 mg/kg) diluted in endotoxin-free castor oil for 5 days before infection experiments. Before the infection with UPEC, a group of mice treated with CsA received an intraperitoneal injection of 2 nmol of anti-Let-7i per mouse. The results showed that 24 h after infection, the bacterial load in the kidneys of CsA-treated mice increased compared to non-treated mice, but interestingly the additional injection of an anti-Let-7i decreased the bacterial load compared to mice only treated with CsA (Fig. 6a). The same results were observed in the bladder of the infected mice (online suppl. Fig. 5). Histological studies showed that the injection of an anti-Let-7i protects against the deleterious effects of CsA during UPEC infection by reducing tissue damage (Fig. 6b). Moreover, the injection of an anti-Let-7i restored the MPO activity inhibited by CsA (Fig. 6c), as well as the secretion of CXCL1 and CXCL2 (Fig. 6d). As a control, we checked the expression of Let-7i in the kidney of mice treated with CsA and injected with the anti-Let-7i. The results showed a decrease of the expression compared to the mice treated with CsA only (Fig. 6e, left panel). The consequences of the injection of the anti-Let-7i on *Tlr4*, *Cxcl1*, and *Cxcl2* mRNA expression were analysed. The results showed that the anti-Let-7i restored the expression of *Tlr4*, *Cxcl1*, and *Cxcl2* when mice were treated with CsA (Fig. 6e, central and right panels). Overall, the results showed that the injection of an anti-Let-7i mimics the effects of CsA in renal epithelial cells during UPEC infection by restoring *Tlr4* expression, *Cxcl1* and *Cxcl2* expression and protein secretion, and MPO activity, and favours the decrease of UPEC bacterial load and kidney damage.

Discussion

We analysed the effects of CsA, a potent immunosuppressant that has wide clinical applications for autoimmune disorders and the prevention of rejection in organ transplantation, on the activation of collecting duct cells that represent the preferential site of adhesion and translocation of ascending UPEC during kidney infections. CsA induced the concentration-dependent inhibition of LPS-stimulated proinflammatory mediators in collecting duct cells. This effect appears to be related to TLR4 down-regulation by the induction of an miR, Let-7i, due to the presence of CsA. Several studies already showed that CsA can specifically induce miRs in different cell types, such as cardiomyocytes, glioma cells, and renal tubular epithelial cells [34–37]. CsA binds to cyclophilin and the cyclophilin-CsA complex will then bind to calcineurin, a protein phosphatase 2B, PP2B, and inhibit IL-2 synthesis, and thereby T-cell functions. However, CsA may have adverse actions and induce tubulointerstitial nephropathy, which could impair the function of renal grafts [38]. Several studies have shown that CsA may induce the apoptosis of renal tubule epithelial cells [14, 39]. These results suggest that CsA may act as a potent cellular stress inducer. However, the effects of CsA on the activation of the different cellular pathways leading to cell survival or apoptosis are still not well known. Prolonged immunosuppression favours the occurrence of UTI and acute pyelonephritis in kidney-transplanted patients. Although UPEC adhering to renal collecting duct cells trigger a solid inflammatory response for the defense of the host, the effects of CsA on immune response capacities resulting from the cellular activation and their consequences on renal bacterial invasion still remain poorly understood. It has already been shown that miR-494 promotes cyclosporine-induced nephrotoxicity in the kidney and epithelial to mesenchymal transition by inhibiting PTEN [35]. Also, CsA induces cardiomyocyte apoptosis through the miR-377-XIAP/NRP2 axis [37]. We demonstrated here, for the first time, the modulation of specific miR by CsA, involved in the regulation of the inflammation, and their direct effects on TLR signaling.

The mechanisms involved in the induction of the expression of miR induced by CsA seem to be related to the activation and the translocation of NFAT, as well as the activation of PKC. The use of a PKC inhibitor alone was able to restore the inflammatory response against UPEC. It has already been shown that CsA and the PKC inhibitor GF10923X antagonise the effects of the miR-19 family, regulating phenotypes of cardiomyocytes via the suppres-

sion of multiple direct target genes [40]. Moreover, several signaling pathways activated during UPEC infection, including the pathways known to activate the innate immune response, interact with calcium-dependent signaling pathways. Interestingly, expression of IL-8 has been shown to be under the control of artificially induced Ca^{2+} oscillations due to the frequency-modulated expression of the transcription factor NF- κ B [41]. Altogether, these findings suggest that CsA induces the transcription of specific miRs through a mechanism dependent of PKC and NFAT, leading to the modulation of the immune response against UPEC.

Analysis of the innate immune response using an experimental model of ascending UTI revealed that the pretreatment of mice with CsA prior to the transurethral inoculation of UPEC induced a marked fall in the cytokine production by epithelial cells as well as in neutrophil recruitment. A dramatic rise in the renal bacterial burden in the infected kidneys of TLR4-expressing C3H/HeN mice, but not in infected TLR4-defective C3H/HeJ mice kidneys, was also shown. The same effect was observed in CsA-treated C3H/HeN-infected kidneys, where the expression of Let-7i was increased. Treatment with a synthetic Let-7i mimic reproduced the effects of CsA. Conversely, treatment with an anti-Let-7i antagonised the effects of CsA and rescued the innate immune response of collecting duct cells against UPEC. It was previously shown that Let-7 family members can modulate TLR4 signaling. Let-7i regulates TLR4 expression in cholangiocytes and contributes to epithelial immune responses against *C. parvum* infection [34]. Another study showed that the expression of Let-7i is associated with the TLR4 signal in coronary artery disease, and statins downregulate the signal via Let-7i expression in these patients [42]. Overall, these results showed that Let-7i is able to directly target the innate immune receptor TLR4 and to quickly modulate the innate immunity against bacteria. Targeting Let-7i seems to be a good way to activate or inhibit the inflammation to favour the resolution of the infection.

UTIs and acute pyelonephritis are the most frequent infectious complications occurring after renal graft. They frequently occur immediately after surgery and are generally considered a consequence of a direct exposure to pathogens favoured by the insertion of an intraurethral stent and urethral catheters. Although antibiotic prophylactic treatments and progress in surgical procedures have reduced the frequency of immediate postoperative UTIs, their frequency still remains higher in the population of kidney-transplanted patients than in the normal

population. Until recently, the impact of acute pyelonephritis on renal graft survival as well as renal graft function has been neglected. A retrospective study on a large cohort of transplanted patients (approx. 29,000 patients) revealed that late UTI occurring after the first post-transplant year represented a risk factor for renal graft dysfunction [3]. We also showed that acute pyelonephritis represents a risk factor, independent of rejection episodes, impairing the long-term kidney graft function [6].

During bacterial invasion, PMNs attracted to the site of infection by chemoattractant chemokines (mainly IL-8 in humans) play key roles in the renal clearance of bacteria [43]. We found that CsA, in addition to its inhibitory action on the TLR4-mediated production of chemoattractant chemokines, also inhibited the expression of nucleotide-binding oligomerisation domain 1 (Nod1), an intracellular receptor involved in the innate immune response against bacteria, in phagocytic PMNs [16]. A primary or acquired deficit in pattern recognition molecules from activated signaling pathways involved in the migration of PMNs to the site of inflammation to kill bacteria leads to functional alterations of PMNs, thus favouring bacterial infections and increasing the susceptibility of the host to repeated infections [16, 44, 45]. This scenario is quite frequently encountered in transplanted patients, who often exhibit recurrent UTI and acute pyelonephritis (representing more than 15% of patients with UTI) under prolonged immunosuppressive treatment [6]. Altogether, these results highlight the inhibitory effect of CsA on the innate immune response, and particularly on the direct effect of CsA on the expression of innate immune receptors.

This study has provided new insights in the understanding of the inflammatory response from injured kidneys colonised by UPEC. In particular, the experiments proposed should further assess the critical role of epithelial renal cells in the recognition of uropathogenic bacteria and their participation, additional to bone marrow-derived cells, in the initiation of a solid immune response for the defense of the host. The demonstration of the mechanism of action of immunosuppressive agents on the inhibition of the inflammatory pathway in target renal epithelial cells is novel and has pathophysiological implications. miRs might be of clinical use in the treatment of infections of the urinary tract and kidneys, especially in transplanted patients, even though the direct effects on allograft rejection has to be checked. A better understanding of the dysregulatory mechanisms of host defense against pathogens evidenced in kidney-transplanted patients is also important and has direct medical implications. The prevention of early UTI and acute py-

elonephritis will certainly permit a prolonged preservation of the renal graft function. Clear identification of dysregulation of some of the immune receptors in blood samples from transplanted patients should also provide the basis for simple tests to control the capacities of immune responses of kidney-transplanted patients subjected to UTIs.

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Disclosure Statement

The authors declare no conflicts of interest.

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