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Lipocalin 2 Imparts Selective Pressure on Bacterial Growth in the Bladder and Is Elevated in Women with Urinary Tract Infection

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Competition for iron is a critical component of successful bacterial infections, but the underlying in vivo mechanisms are poorly understood. We have previously demonstrated that lipocalin 2 (LCN2) is an innate immunity protein that binds to bacterial siderophores and starves them for iron, thus representing a novel host defense mechanism to infection. In the present study we show that LCN2 is secreted by the urinary tract mucosa and protects against urinary tract infection (UTI). We found that LCN2 was expressed in the bladder, ureters, and kidneys of mice subject to UTI. LCN2 was protective with higher bacterial numbers retrieved from bladders of *Lcn2*-deficient mice than from wild-type mice infected with the LCN2-sensitive *Escherichia coli* strain H9049. Uropathogenic *E. coli* mutants in siderophore receptors for salmochelin, aerobactin, or yersiniabactin displayed reduced fitness in wild-type mice, but not in mice deficient of LCN2, demonstrating that LCN2 imparts a selective pressure on bacterial growth in the bladder. In a human cohort of women with recurrent *E. coli* UTIs, urine LCN2 levels were associated with UTI episodes and with levels of bacteriuria. The number of siderophore systems was associated with increasing bacteriuria during cystitis. Our data demonstrate that LCN2 is secreted by the urinary tract mucosa in response to uropathogenic *E. coli* challenge and acts in innate immune defenses as a colonization barrier that pathogens must overcome to establish infection. ***The Journal of Immunology*, 2014, 193: 6081–6089.**

Uncomplicated urinary tract infections (UTIs) are common in otherwise healthy individuals. Half of all women will get one or more UTIs before reaching their mid-30s, and recurrent infections are frequent also in women without any anatomical abnormalities in the urinary tract (1, 2). Uropathogenic bacteria access the urinary tract through the urethra and subsequently colonize the bladder. Although urine is usually sterile, asymptomatic bacteriuria (ASB) at $\geq 10^5$ CFU/ml is found in ~5–10% of healthy, premenopausal women (3). UTIs, accompanied by inflammation and symptoms, include cystitis when infection is limited to the bladder and pyelonephritis when bacteria ascend the ureters to infect the kidneys. In the mouse model of UTI, to colonize the host, uropathogenic bacteria first adhere to and invade the cells of the urinary mucosal epithelium (urothelium) where they can replicate into transient, intracellular bacterial communities (4), and later can flux out into the lumen of the bladder again. Interaction with urothelial cells induces a local inflammatory response mediated, at least partly, by TLRs (5–8).

Recurrent UTI (rUTI) is a common syndrome in otherwise young healthy women. Previous studies suggest that 27–44% of women who experience an initial UTI develop rUTI (2). Behavioral factors, such as sexual intercourse and spermicide use, are strongly associated with an increased risk of rUTI (9, 10). However, many women with increased susceptibility to UTIs do not have obvious behavioral or anatomic risk factors, suggesting that additional host or bacterial factors may be present. We previously identified an association between TLR polymorphisms and risk of rUTI (11, 12). rUTI could result from repeated infections by bacteria ascending the bladder from the intestine, vagina, or periurethra, although more recent studies suggest that uropathogenic *Escherichia coli* (UPEC) can form persistent reservoirs within the bladder epithelium and later emerge to cause reinfections (4, 13, 14). Despite the high prevalence and impact on society, the pathogenesis of UTI and risk factors for persistent infection or recurrence are poorly understood.

To successfully colonize the urinary tract, UPEC strains express a number of virulence factors, including fimbriae, flagella, toxins, and various iron uptake systems (15–17). It is presently not clear,

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T.H.F., M.S., and T.R.H. conceived and planned the study; M.S., M.H., A.M., and T.H.F. performed the experiments; R.K.S., S.D.H., and H.L.T.M. provided WT and mutant rLcn2 or UPEC strains, respectively; results were analyzed by M.S., A.S., J.K.D., H.L.T.M., T.M.H., T.R.H., and T.H.F.; P.L.R. performed statistical analyses; and M.S., P.L.R., T.R.H., and T.H.F. wrote the paper. All authors discussed the results and commented on the manuscript.

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Abbreviations used in this article: ASB, asymptomatic bacteriuria; LB, Luria broth; LCN2, lipocalin 2; LF, lactoferrin; rUTI, recurrent UTI; uLCN2, urinary LCN2; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infection.

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however, how antibacterial host defenses influence the virulence and colonization of the urinary tract by UPEC. We previously demonstrated that lipocalin 2 (LCN2) is an innate immunity protein that works by starving bacteria for iron (18). LCN2 colocalizes with lactoferrin (LF) in specific granules of neutrophils and is induced in epithelial cells and macrophages in response to inflammatory stimuli (19, 20). Upon encountering invading bacteria the TLRs on immune cells stimulate a massive induction and secretion of LCN2 that, in turn, limits bacterial growth by sequestering iron-laden siderophores, small iron-sequestering molecules secreted by bacteria in response to iron limitation (17, 18, 21). The high affinity for iron allows siderophores to extract iron from host proteins with subsequent uptake by the pathogen through specific receptors (17, 22). Siderophores are classified according to their chemistry of iron chelation, and LCN2 typically limits the growth of bacteria that depend on catecholate siderophores for iron acquisition (21). Pathogenic bacteria may use alternate siderophores with different chemistry to escape LCN2-mediated iron deprivation and successfully establish an infection. *Salmonella enterica* serovar Typhimurium escapes LCN2 defenses by glucosylating enterobactin, a catecholate siderophore with high affinity for iron (23, 24). Structurally distinct siderophores may also be expressed when bacteria need to adapt to environmental changes that will impact the iron-binding affinity of the siderophores (22, 25). Siderophores are among several virulence factors that are critical for bacteria to successfully establish a UTI (26–28). We hypothesize that LCN2 plays a role in protection of the urinary tract from bacterial infection. Using a mouse model of ascending UTI and a cohort of women with recurrent *E. coli* UTI, we found that the urinary tract mucosa secretes LCN2 in response to UPEC challenge, with urinary LCN2 (uLCN2) levels temporally reflecting the severity of the insult. We also demonstrate that LCN2 imparts a selective pressure on UPEC growth in the bladder favoring strains expressing alternative iron acquisition systems. LCN2 is thus rapidly induced throughout the urinary tract upon bacterial challenge and serves in local defense of the urinary tract mucosa.

Materials and Methods

Histology

Organ samples were fixed in buffered formalin, processed through standard paraffinization overnight, cut in 5-μm-thick sections, and stained with H&E. For immunostaining, sections were deparaffinized and subjected to Ag retrieval at pH 6, blocked, and incubated overnight with Abs against LCN2 (18) or neutrophils (Ly-6G/Gr-1clone RB6-8C5, eBioscience) followed by biotinylated secondary Ab (goat anti-rabbit, Dako, 1:200) and LSAB2 streptavidin–HRP (Dako). Sections were resolved using the EnVision DAB detection system (Dako).

ELISA and quantitative real-time PCR

Cytokines levels were quantified by ELISA analysis from human urine samples (NGAL ELISA by R&D Systems) or homogenized mouse bladders (18, 29). For quantitative real-time PCR analysis, tissue samples from bladder and kidney, stored on RNA*later* RNA stabilization reagent (Qiagen), were homogenized by bead-beating with a FastPrep-24 instrument (MP Biomedicals). Total RNA was isolated in an automated protocol using the RNeasy mini kit with DNase I digestion on a QIAcube instrument (all Qiagen). After cDNA synthesis (high-capacity reverse transcription kit, Applied Biosystems), quantitative real-time PCR was performed on a StepOnePlus PCR system using TaqMan fast advanced master mix and TaqMan gene expression assays (Applied Biosystems: LCN2 [Mm01324470_m1], LF [Mm00434787_m1], CXCL1 [Mm00433859_m1], CCL2 [Mm00441242_m1], IL-1β [Mm01336189_m1], IL-6 [Mm00446190_m1], TNF [Mm00443258_m1], IL-10 [Mm00439614_m1], IL-17a [Mm00439618_m1], and IL-22 (Mm01226722_g1]). Gene expression was normalized to GAPDH and β-actin as endogenous controls (Applied Biosystems: GAPDH [Mm99999915_g1] and β-actin [Mm00607939_s1]), and relative quantification values to gene expression in uninfected mice were calculated with StepOne software version 2.1 (Applied Biosystems).

Bacterial strains, determination of virulence fators, and culture conditions

E. coli strains used in the mouse experiments are the same strains used by Flo et al. (18) (H9049) and Garcia et al. (26). For the in vitro experiments in Supplemental Fig. 2, the *ybtS iroB* double mutant of *E. coli* 536 was constructed as described in Garcia et al. (26) using the λ Red recombinase system. In short, kanamycin or chloramphenicol resistance cassettes were PCR amplified from the template plasmid pKD4 or pKD3, respectively, using primers containing regions identical to the 5′ and 3′ ends of the gene to be deleted (primers: *ybtS*, forward, 5′-GAATTTCTACATCTGGCGTTACCAGAGGAACAATGGCTACGTGTAGGCTGGAGCTGCTTC-3′, reverse, 5′-CCATTAAATAGGGCGCAATGCTCGCTAATTTCTCCCGGGATGGGAATTAGCCATGGTCC-3′; *iroB*, forward, 5′-GGGGGCTGGCAAATGATTATCATGAAGGTGTAGTGGTGTAGGCTGGAGCTGCTTC-3′, reverse, 5′-ATCTGGTAAAGCAGGTTTAACGAAAAGTCGTGTCCTGCTGCTGGCATATGCATAGAATA-3′). The resulting product was transformed into *E. coli* 536 and replaced >80% of the target gene by homologous recombination. All mutants were confirmed by PCR. Virulence factors of clinical strains were determined by PCR as described earlier (30) using the following primers: *fyuA*, forward, 5′-TGATTAACCCCGCGACGGGAA-3′, reverse, 5′-CGCAGTAGGCACGATGTTGTA-3′; *iutA*, forward, 5′-GGCTGGACATCATGGGAACTGG-3′, reverse, 5′-CGTCGGGAACGGGTAGAATCG-3′; *iroN*, forward, 5′-AAGTCAAAGCAGGGGTTGCCCG-3′, reverse, 5′-GACGCCGACATTAAGACGCAG-3′; *papAH* (hemolysin), forward, 5′-ATGGCAGTGGTGTCTTTTGGTG-3′, reverse, 5′-CTGCCCCACCATACGTCTCTTC-3′; *fimH*, forward, 5′-TGCAGAACGGATAAGCCGTGG-3′, reverse, 5′-GCAGTCACCTGCCCTCCGGTA-3′; *papG*, forward, 5′-CTGTAATTACGGAAGTGATTTCTG-3′, reverse, 5′-ACTATCCGGCTCCGGATAAACCAT-3′. Bacteria were grown at 37°C with aeration in Luria broth (LB; 10 g/l trypyone, 5 g/l NaCl, and 5 g/l yeast extract) unless otherwise stated. Antibiotics were added at appropriate concentrations (100 μg/ml ampicillin, 25 μg/ml kanamycin and 20 μg/ml chloramphenicol) (26).

Mouse cochallenge model of ascending UTI

All animal work was approved by the local Animal Care Committee (reference no. FOTS 640). C57BL/6 wild-type mice and *Lcn2*-deficient mice (18) were inoculated transurethrally with mixed cultures of UPEC mutants as previously described (26). Bacteria were cultured statically at 37°C in LB without antibiotics for 24 h, rediluted 1:1000, and grown statically for further 24 h prior to inoculation. Two, three, or four UPEC strains were mixed in equal ratios, and 50 μl (10^8 CFU) was delivered to each mouse via a sterile 0.28-mm polyethylene catheter attached to a syringe (31). Mice where sacrificed at 6 h, 18 h, 4 d, or 7 d after infection, and bladder, ureters, and kidneys were collected. Left ureters and kidneys were fixed for histochemistry and a 3-mm punch biopsy was used for mRNA analyses. Tissue homogenates were serially diluted and plated onto LB agar containing appropriate antibiotics to enumerate CFU per tissue. For multistrain coinfections, a fitness index for each organ sample was calculated by dividing the fraction of each mutant in the output by the fraction of each mutant in the inoculum (26).

Study subjects and study design of human clinical study

The study design and inclusion of study subjects are described in Czaja et al. (30). From January 2003 through December 2006, premenopausal women aged 18–49 y with a self-reported history of at least one UTI during the past year and a current diagnosis of acute cystitis (dysuria, frequency, or urgency with a concentration of a uropathogen in urine $\geq 1 \times 10^2$ CFU/ml) were recruited at the student health center of the University of Washington. Exclusion criteria included known anatomic or functional abnormalities of the urinary tract, chronic illness requiring medical supervision, pregnancy or planned pregnancy during the next 3 mo, and symptoms or signs of acute pyelonephritis. *E. coli* rUTI was defined as the presentation of a subject to the clinic for medical evaluation of symptoms of acute cystitis (dysuria, frequency, or urgency) with a concentration of *E. coli* in urine 1×10^2 CFU/ml, regardless of the enrollment UTI urine isolate. When *E. coli* was not isolated, the organism present in the urine at the greatest quantity was considered to be the causal uropathogen. Urine samples were available for the cystitis episode at the time of enrollment for all women and for an additional 49 recurrent UTI episodes, which occurred during the 90-d follow-up period in 36 women. The median age was 22 y, with 88% having a history of three or more lifetime UTIs including a median of three in the year before enrollment. Midstream urine specimens were collected on the day of rUTI and surrounding days and stored at −70°C for later testing of LCN2 levels. Clean-catch midstream urine specimens were collected in a sterile container at the clinic or in a sterile BD Vacutainer urine tube (Becton Dickinson) containing a lyophilized preservative including boric acid and sodium formate at home. All uropathogens present in midstream urine at ≥1 × 10^2 CFU/ml in clinic specimens and at ≥1 × 10^3 CFU/ml in home specimens were identified and quantified. The study design was approved by the Human Subjects Review Committee at the University of Washington, and all subjects provided written informed consent.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 and 6 and SAS 9.3. For multigroup comparisons, the Kruskal–Wallis test was used with a Dunn procedure for pairwise comparisons. Otherwise, the Wilcoxon signed-rank test was used for paired observations, and the Wilcoxon rank sum test was used to compare unpaired observations.

Results

LCN2 is expressed in the urinary tract upon urinary tract infection

LCN2 is induced in gut and airway mucosal surfaces upon infection (24, 32). To investigate whether LCN2 is similarly induced in the urinary tract upon infection, C57BL/6 wild-type mice were inoculated transurethrally (31) with PBS or the clinical UPEC isolate, CFT073.

Increasing levels of LCN2 protein were measured in wild-type bladder and kidney homogenates 6 h to 4 d postinoculation (Fig. 1A). At 6 h, LCN2 mRNA was 27-fold upregulated in bladders and 6-fold upregulated in kidneys of inoculated mice relative to control mice (Fig. 1B). However, 4 d postinoculation kidney LCN2 expression far exceeded that of the bladders.

Although CFU varied greatly as is typical, LCN2 induction tended to mirror bladder and kidney colonization: at 6 h postinoculation all organs were colonized and LCN2 was elevated (Fig. 1C, 1D). Mice that were still infected (CFU^{hi}) 1 and 4 d later showed persistent and even increased LCN2 protein and mRNA levels compared with mice that later cleared the infection (CFU^{lo}). Taken together, our results indicate that LCN2 was induced locally in the urinary tract and kidney epithelium by CFT073 challenge.

UPEC colonization and invasion of the urothelium activates an acute inflammatory response that can be protective or predisposes the animal to chronic infection depending on the magnitude (33). We therefore examined whether there was a general difference between wild-type and *Lcn2*-deficient mice in their acute inflammatory response upon bladder insult by measuring expression of inflammatory chemokines central in recruitment of neutrophils (CXCL1) or monocytes (CCL2), inflammatory cytokines (IL-1β, TNF, IL-6, IL-10, IL-17a, IL-22), and the antibacterial iron-binding protein LF 6 h postinoculation. Levels of inflammatory

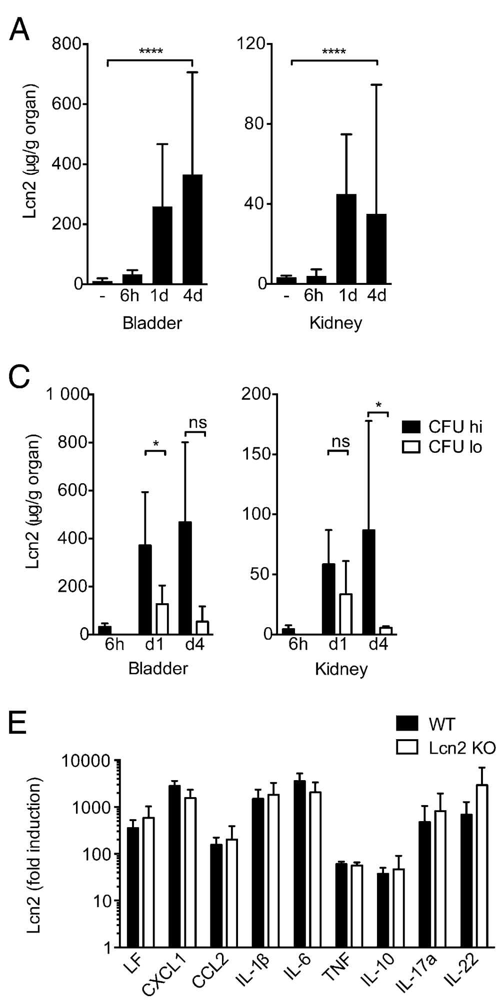

FIGURE 1. LCN2 is expressed in the urinary tract during UTI. C57BL/6 wild-type and *Lcn2*-deficient mice were transurethrally inoculated with UPEC strain CFT073 for the indicated period of time before bladders and kidneys were harvested and analyzed for transcript levels by quantitative RT-PCR or protein production by ELISA. mRNA levels are given as fold induction over uninfected control organs; protein levels are given as micrograms per gram of organ. LCN2 protein levels (**A**) and mRNA levels (**B**) in bladders and kidneys of wild-type mice 6 h (n = 15), 1 d (n = 10), and 4 d (n = 9) postinoculation are shown. LCN2 protein levels (**C**) and mRNA levels (**D**) in bladder and kidney 6 h, 1 d, and 4 d postinfection are grouped according to high (CFU^{hi} > 1000 CFU) or low/absent (CFU^{lo} < 1000 CFU) bladder colony plating. (**E**) mRNA levels of LF, chemokines, and cytokines in bladders of wild-type and *Lcn2*-deficient mice 6 h postinfection with UPEC CFT073. Data represent means and SDs for n mice as indicated. *$p < 0.05$ Wilcoxon signed-rank test, ****$p < 0.0001$ Kruskal–Wallis test.

cytokines were not significantly different in wild-type and *Lcn2*-deficient mice (Fig. 1E). The expression of IL-6 and CXCL1 was by far the highest (almost 3000-fold upregulated) followed by IL-1β, whereas TNF, IL-10, and CCL2 were less induced. Leukocyturia increased with infection in mice of both genotypes, but no differences were observed between genotypes (Supplemental Table I). It has previously been shown that secretion of LCN2 by epithelial cells may require IL-17 or IL-22, most likely secreted from retinoic acid–related orphan receptor γt-expressing cells such as innate lymphoid cells or γδT cells (24, 34, 35). We observed a dramatic increase in transcript of both IL-17a and IL-22 in UPEC-infected bladders 6 h postinoculation (Fig. 1E).

LCN2 is expressed by infiltrating neutrophils and urothelial cells in UPEC-infected mice

To identify the cellular sources of LCN2 in the urinary tract, we infected wild-type and *Lcn2*-deficient mice with CFT073 and examined the production of LCN2 in situ 1 d postinoculation using immunohistochemistry. LCN2 was highly present in both infiltrating neutrophils and in superficial epithelial cells facing the lumen of the bladder, similar to what has been observed in inflamed intestinal and airway epithelium (Fig. 2A) (24, 32, 36). Neutrophils were evident from their polymorphic/multilobular nuclei and positively confirmed by Ly6G staining (not shown). LCN2 was also produced in kidney tubular cells as previously shown by Paragas et al. (37) (Fig. 2B), as well as in the urothelium of ureters (Fig. 2C). Alternatively, LCN2 was not induced in the bladders, ureters, or kidneys harvested from mock-infected mice or *Lcn2*-deficient mice (Supplemental Fig. 1). Thus, our results indicate that LCN2 is produced throughout the urinary tract during UTI from infiltrating neutrophils and in the bladder, ureter, and kidney epithelium.

LCN2 imparts selective pressure on bacterial growth in the bladder favoring strains expressing more LCN2-insensitive iron acquisition systems

Our main hypothesis is that LCN2 is rapidly secreted in response to microbial insults and imparts a selective pressure on colonization of

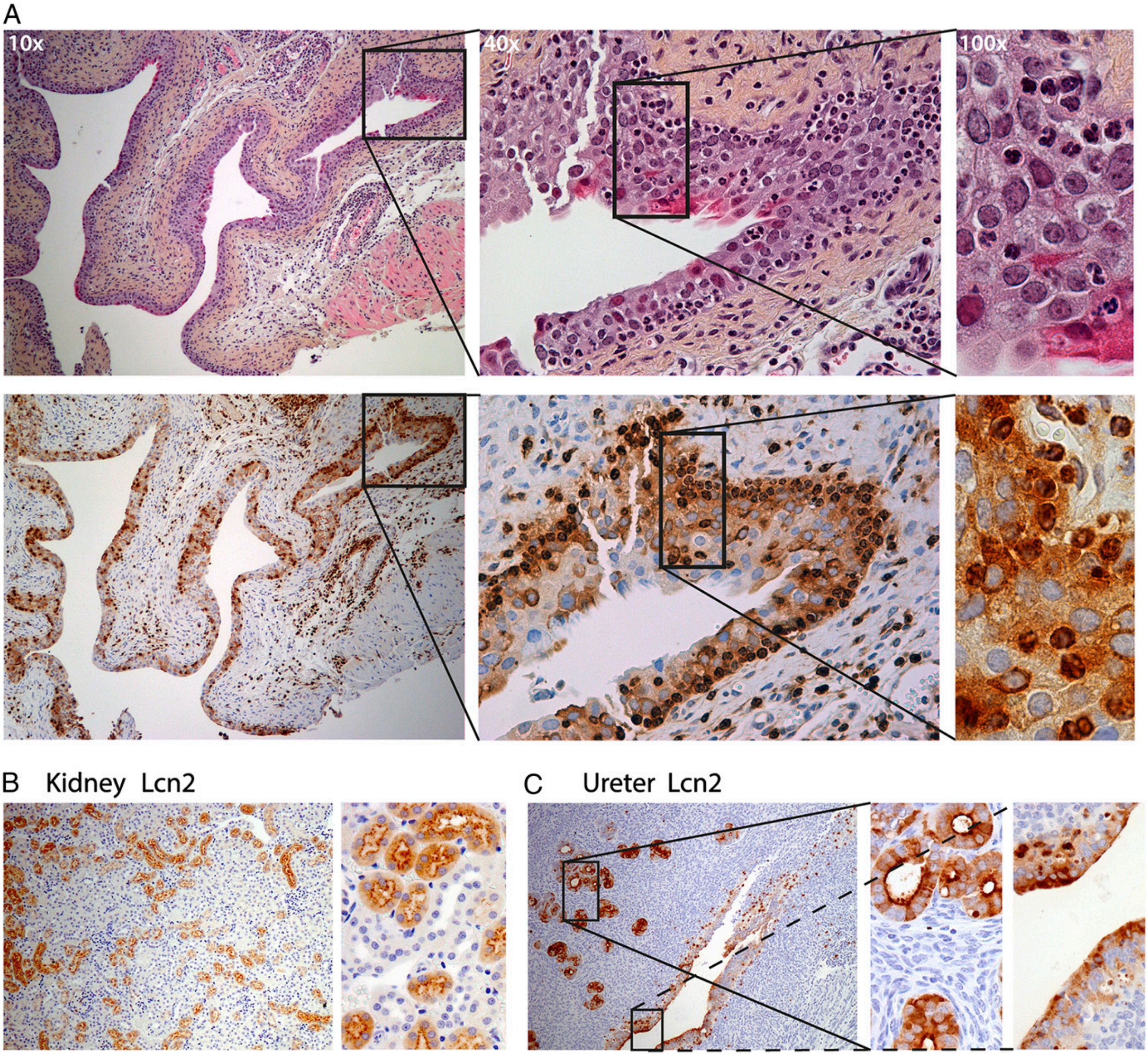

FIGURE 2. LCN2 is expressed in epithelial cells and granulocytes of infected bladders. C57BL/6 wild-type mice were inoculated transurethrally with UPEC strain CFT073 for 1 d before bladders, ureters, and kidneys were analyzed. (**A**) H&E and LCN2 immunohistological staining of bladders. (**B** and **C**) Immunohistochemical staining of LCN2 in kidney and ureter (original magnification 10× [*left*] with 40× inserts [*right*]).

mucosal surfaces, including the urinary tract. The expression of alternative iron acquisition systems not inhibited by LCN2 would thus represent a central virulence trait for mucosal pathogens. To investigate whether LCN2 actually protects against UTI, we transurethrally instilled *E. coli* strain H9049 (18) into wild-type mice and *Lcn2*-deficient mice. *E. coli* H9049 utilizes enterobactin for obtaining iron, a siderophore recognized by LCN2. Three days later, we sacrificed the mice and homogenized the bladders for CFU enumeration. Although both mouse strains had bacteria present in their bladders, the LCN2-deficient mice had significantly higher bacterial loads 3 d postinoculation (Fig. 3A).

In contrast to the nonpathogenic *E. coli* H9049, most UPEC strains can make and use siderophores that are not bound by LCN2 (27). Indeed, UPEC strain CFT073 has the ability to make salmochelin and aerobactin (26, 38), and strain 536 makes salmochelin and yersiniabactin (39) in addition to enterobactin. We next compared the in vitro growth of *E. coli* H9049, UPEC 536, and a mutant of UPEC 536 deficient in salmochelin and yersiniabactin (dd536) in the presence of recombinant human LCN2 or LCN2KK, a mutant unable to bind siderophores (R.K. Strong, unpublished data). As expected, rLCN2 inhibited the growth of H9049 and dd536 (both enterobactin-dependent), but not the 536 wild-type strain, whereas the recombinant Lcn2KK mutant did not affect the growth of either strain (Supplemental Fig. 2). These results demonstrate the specificity of LCN2 for enterobactin, and also that UPEC 536 is resistant to LCN2 during in vitro growth.

E. coli without a functional salmochelin receptor, IroN, is unable to use the siderophore salmochelin (40), and IroN mutants are outcompeted by wild-type bacteria in mice (41). To investigate whether selection pressure by LCN2 can account for the decreased fitness observed for the IroN mutant, we transurethrally instilled a 1:1 mixture of wild-type CFT073 and CFT073 Δ*iroN* into C57BL/6 wild-type mice and *Lcn2*-deficient mice. Three days later, we sacrificed the mice and homogenized the bladders for CFU enumeration. The IroN mutant was significantly outcompeted in the wild-type mouse (Fig. 3B) but not in *Lcn2*-deficient mice, suggesting that salmochelin confers a modest growth advantage in the presence of LCN2.

We recently showed that IutA-mediated uptake of aerobactin and FyuA-mediated uptake of yersiniabactin contributed more than salmochelin to UPEC iron uptake during mouse UTI (26). Next, we thus compared the fitness of CFT073 Δ*iutA* to mutants in hydroxamate siderophores (Δ*fhyA*, Δ*fhuE*) in UTI infection of wild-type and *Lcn2*-deficient mice. The IutA mutant was outcompeted in the wild-type mice whereas all mutant strains showed equal fitness in *Lcn2*-deficient mice, and the fitness of the IutA mutant was significantly higher in the *Lcn2*-deficient mouse than in wild-type mice (Fig. 3C). We next inoculated wild-type and *Lcn2*-deficient mice with a mixture of 536 mutants Δ*fyuA*, Δ*fhyA*, Δ*fhuE*, and a double mutant in heme uptake (Δ*hma*Δ*chuA*). As for the CFT073 aerobactin mutant, the 536 yersiniabactin mutant *fyuA* showed significant reduction in fitness compared with mutants in *fhuA* and the

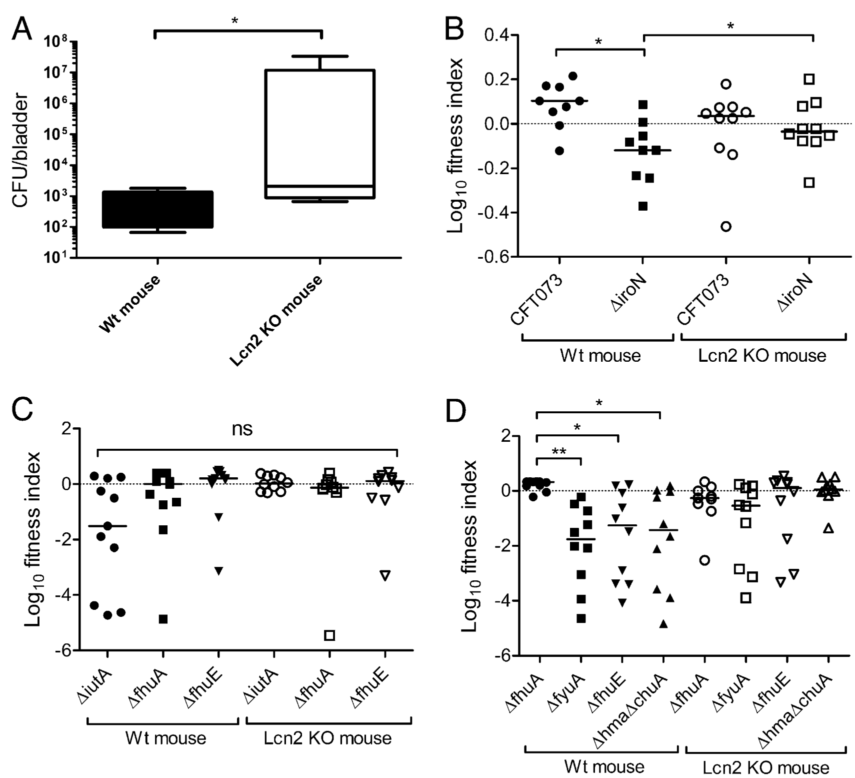

FIGURE 3. LCN2 protects against UTI and causes loss of fitness in mutants of UPEC unable to use salmochelin, aerobactin, or yersiniabactin. Infection of C57BL/6 wild-type and *Lcn2*-deficient mice with nonpathogenic and LCN2-sensitive *E. coli* H9049 and competition experiments with mutant strains of UPEC CFT073 and 536 defective in genes of siderophore uptake systems are shown. Fitness indices were calculated by dividing the fraction of each mutant in the output (CFU/tissue or CFU/ml culture) by the fraction of each mutant in the input (CFU/ml inoculum). (**A**) *E. coli* H9049 infection in C57BL/6 wild-type and *Lcn2*-deficient mice. Mice were sacrificed at 72 h, bladder homogenates were plated, and colonies were enumerated. Data are shown as box-and-whisker graphs with whiskers showing minimum and maximum values. (**B**) In vivo competition with a 1:1 mix of CFT073 wild-type and a mutant unable to use salmochelin (Δ*iroN*) in C57BL/6 wild-type and *Lcn2*-deficient mice. Mice were sacrificed at 72 h, bladder homogenates were plated, colonies were enumerated, and the fitness index was calculated. (**C**) In vivo competition with a 1:1:1 mixture of UPEC CFT073 mutants in genes for hydroxamate siderophore receptors (Δ*fhuA* and Δ*fhuE*) or aerobactin (Δ*iutA*). (**D**) In vivo competition with a 1:1:1:1 mixture of mutants in UPEC 536 [same as in (A)]. Results for all mice in all experiments are shown (n = 9 or 10). Line represents the median. In (A), the Wilcoxon rank sum test was used (*$p < 0.05$). In (B) and (D), the Kruskal–Wallis test and the Dunn procedure were used. For (B) and (D), the Kruskal–Wallis test showed significance ($p < 0.05$). *$p < 0.05$, **$p < 0.01$ by the Dunn procedure. In (C), the Kruskal–Wallis test was not significant (p = 0.06) despite the individual comparisons showning significance ($p < 0.05$).

Table I. uLCN2 levels in women before and during cystitis episodes

Cystitis Window and Groups[a]	N	Median (ng/ml)	75th Percentile (ng/ml)	90th Percentile (ng/ml)	Maximum (ng/ml)
>14 d Prior to cystitis					
No ASB ($< 10^3$)	209	12.8	29.2	54.4	1636.6
Medium ASB (10^3 to $<10^5$)	27	34.2	62.1	133.4	307.4
High ASB ($\geq 10^5$)	35	55.6	97.5	144.5	254.8
14 d Cystitis window[b]					
4–14 d Prior to cystitis	30	21.6	31.8	71.7	206.4
1–3 d Prior to cystitis	64	22.9	48.4	128.6	1034.3
Cystitis	96	241.0	507.4	973.2	2489.0

[a]Cystitis category includes non–*E. coli* episodes. ASB is *E. coli* only.
[b]The 14-d period leading up to and including the day of cystitis diagnosis.

hma chuA double mutant in wild-type mice, but not in *Lcn2*-deficient mice (Fig. 3D). In fact, all constructs with reduced fitness in the wild-type mouse regained fitness in the *Lcn2*-deficient mouse. Taken together, our results suggest that LCN2 dictates siderophore redundancy, and that aerobactin and yersiniabactin represent a selective advantage for colonization of the urinary tract when LCN2 is present more so than does salmochelin.

LCN2 levels rise during cystitis episodes

Given the rapid and pronounced induction of LCN2 in the urinary tract of infected mice, we investigated the clinical relevance of these findings in humans. uLCN2 was assayed in samples collected from 104 women with acute cystitis and a history of UTI within the previous year during a 3-mo follow-up period (30). *E. coli* was the most frequently isolated uropathogen (50 nonhemolytic and 31 hemolytic *E. coli*, 4 other Gram-negative species, 5 *Staphylococcus saprophyticus*, and 2 other Gram-positive species). uLCN2 levels were elevated during episodes of cystitis (241.0 ng/ml) in comparison with asymptomatic periods with $<10^3$ CFU/ml (12.8 ng/ml) (Table I). LCN2 levels were highest in samples with hemolytic or nonhemolytic *E. coli* or Gram-negative uropathogens (295.5, 246.4, and 177.8 ng/ml, respectively) in comparison with *S. saprophyticus* or other Gram-positive pathogens (94.0 and 76.3 ng/ml, respectively). We also found a positive correlation between uLCN2 and urine WBC counts or leukocyte esterase in 570 samples (Spearman correlation coefficient of 0.784 and 0.462, respectively, $p < 0.0001$).

We then examined the association of uLCN2 levels with levels of bacteriuria, clinical symptoms, and time before cystitis episodes (Table I). In examining the days remote from a recurrent UTI (i.e., >14 d before), levels of uLCN2 were higher in urine samples with medium (*E. coli* 10^3 to $< 10^5$ CFU/ml) to high (*E. coli* $\geq 10^5$ CFU/ml) ASB (34 and 56 ng/ml) than in urines with $<10^3$ CFU/ml (13 ng/ml). We next examined uLCN2 levels before and during active cystitis episodes infection. uLCN2 levels were higher for patients at the time of clinical diagnosis of cystitis (241 ng/ml) compared with samples taken 1–3 d (23 ng/ml) and 4–14 d (22 ng/ml) prior to the clinical diagnosis of cystitis (Table I). For 14 of the patients, culture data were available for the third day preceding cystitis. A pairwise comparison showed a significant increase in uLCN2 levels at the time of cystitis compared with 3 d prior (Fig. 4, 343 versus 16 ng/ml, $p = 0.0009$). The difference was most pronounced compared with patients who had $<10^3$ CFU/ml on the third day preceding UTI.

In our murine UTI model, as noted above, there was a decrease in bladder LCN2 levels in mice clearing the infection, whereas LCN2 levels persisted in mice still carrying bacteria in their bladders. This was also true for patients in the 14 d after a UTI when levels of LCN2 dropped quickly, with urines growing $<10^3$ CFU/ml ($n = 19$) containing levels ranging from 0.21 to 51.73 ng/ml. Our results show that urine LCN2 levels increase dramatically upon infection of the urinary tract, remain high until infection is cleared, and then drop rapidly after clinical and microbiologic resolution. Our results also suggest that LCN2 can be used as a marker for ASB in humans.

Expression of LCN2 evasive siderophores is associated with higher E. coli *CFUs during cystitis episodes*

To investigate whether there was an association between siderophore expression, uLCN2, *E. coli* CFU, and UTI virulence factors, we analyzed the infecting strains for the presence of genes for siderophore receptors IroN (salmochelin), IutA (aerobactin), and FyuA (yersiniabactin) as well as hemolysin and fimbriae by PCR (Table II). There were 80 UPEC strains from enrollment UTIs for which uLCN2 levels were ascertained. Fifteen of the UPEC strains did not express any of the tested alternative siderophore receptors whereas 35 expressed one, 52 expressed two, and 24 expressed all three siderophore receptors IroN, IutA, and FyuA. Thirty-eight percent of the strains were resistant to ciprofloxacin, amoxicillin/clavulonic acid, or ampicillin, and there was no association to the expression of additional siderophore systems (Table II). We observed a trend that patients with high levels of LCN2 were infected with UPEC strains expressing more than one siderophore systems, although data were not significant (Supplemental Table II). A similar trend was also seen in women with recurrent UTIs (Table II), although we did not detect any difference in the number of siderophore systems when comparing strains from the first and subsequent episodes in patients with rUTI (data not shown). Interestingly, we observed higher *E. coli*

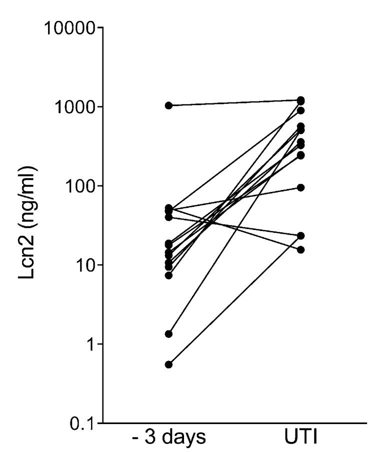

FIGURE 4. Onset of UTI is preceded by a rapid increase in urine LCN2 levels . Pairwise comparison is shown of LCN2 protein levels in urine samples from 14 patients taken 3 d prior to onset of UTI and at cystitis episodes (enrollment UTI or recurrent UTI during the follow-up period). There was a significant increase in uLCN2 from day −3 to UTI events ($p < 0.001$, Wilcoxon signed-rank test).

Table II. Siderophore uptake systems in *E. coli* isolated from UTI patients related to drug resistance, rUTI, and the presence of genes for virulence factors type 1 fimbriae, hemolysin, and P fimbriae

Siderophore[a]	AR[b]	rUTI[c]	Type 1 Fimbriae[d]	Hemolysin[e]	P Fimbriae[f]
0	45 (5/11)	27 (3/11)	100 (11/11)	0 (0/11)	0 (0/11)
+1	23 (5/22)	32 (7/22)	95 (21/22)	18 (4/22)	23 (5/17)
+2	41 (17/38)	42 (16/38)	100 (38/38)	45 (17/38)	32 (12/38)
+3	43 (6/14)	50 (7/14)	100 (14/14)	50 (7/14)	14 (2/14)

[a]Denotes number of alternative siderophores expressed as determined by PCR because all strains are presumed to have enterobactin.
[b]Denotes antbiotic resistance of one or more of ciprofloxacin, augmentin (amoxicillin/clavulanic acid), or amoxicillin shown as percentage of strains (frequency in parentheses).
[c]Denotes rUTI as percentage of strains (frequency in parentheses).
[d]Denotes percentage of strains that express type 1 fimbriae (frequency in parentheses).
[e]Denotes percentage of strains that express hemolysin (frequency in parentheses).
[f]Denotes percentage of strains that express P fimbriae (frequency in parentheses).

CFU levels with increasing numbers of siderophores (Fig. 5, median CFU 2.7×10^4, 4.2×10^4, $\geq 10^5$, and $\geq 10^5$ for 0, 1, 2, and 3 number of iron-acquiring siderophores, respectively, p = 0.002). Taken together, these data suggest that the number of siderophore systems is associated with higher levels of bacteriuria during cystitis episodes.

Discussion

In this study, we show that LCN2 is rapidly and locally produced in the urinary tract in response to bacterial challenges and confers a selective pressure on the type of siderophores required by uropathogenic *E. coli* for establishing UTI in mice. Concordantly, in women experiencing rUTI, the number of siderophore systems was associated with higher levels of bacteriuria during episodes of cystitis. To our knowledge, this is the first demonstration that LCN2 mediates protection from UTIs.

UPEC commonly produces several LCN2-susceptible and -resistant iron acquisition systems for reasons that are not entirely clear (27). LCN2 efficiently sequesters enterobactin, a catecholate siderophore expressed by nearly all *E. coli* strains (18, 23). LCN2 exerts a substantial pressure on UPEC strains to allocate resources for expression of LCN2-resistant iron uptake systems. This could be siderophores that are chemically modified to prevent LCN2 binding (e.g., salmochelin is glucosylated enterobactin), siderophores built on a different chemical backbone (aerobactin, a hydroxamate siderophore; yersiniabactin, a mixed type siderophore), or nonsiderophore iron acquisition systems, such as heme (27). The relative efficiency of these iron acquisition systems is determined by the chemical environment and available iron sources, possibly explaining the need for several seemingly redundant systems as UPEC moves through the urinary tract (16, 22, 28). We previously found in BL/6 wild-type mice that heme and noncatecholate siderophores (yersiniabactin and aerobactin) contribute to UPEC iron acquisition at specific sites during UTI (26). Enterobactin has a substantially higher affinity for iron than do yersiniabactin and aerobactin (17), and presumably it confers an advantage under conditions of LCN2 deficiency or at sites where LCN2 is not expressed. Our findings support this hypothesis, as 1) the nonpathogenic, enterobactin-dependent and LCN2-sensitive *E. coli* strain H9049 could colonize the bladders of *Lcn2*-deficient mice, and 2) none of the LCN2-evasive iron acquisition systems was required for UPEC bladder colonization of *Lcn2*-deficient mice. A similar relationship between siderophore efficiency and LCN2 evasion has been shown for *Klebsiella pneumoniae* (32, 42). However, the expression of salmochelin, but not yersiniabactin, allowed *K. pneumoniae* to evade growth inhibition by LCN2 in human serum, whereas the same siderophores were dispensable for growth in human urine (25, 42).

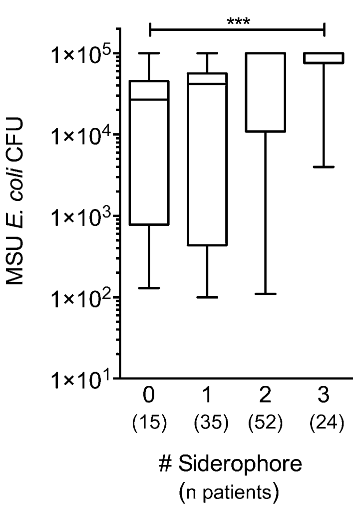

FIGURE 5. Urine *E. coli* levels are associated with expression of alternative siderophores in women with recurrent UTIs. *E. coli* was isolated from MSU from 80 women with recurrent UTI and analyzed for the presence of alternative siderophore receptors *iroN* (salmochelin), *iutA* (aerobactin), and *fyuA* (yersiniabactin) by PCR. Increasing numbers of siderophores were associated with higher levels of bacteriuria (*E. coli* CFU levels) during cystitis episodes. Box-and-whisker graph with whiskers showing minimum and maximum values (***p = 0.002 Kruskal–Wallis test). MSU, midstream urine.

Our findings support a model where LCN2 is rapidly secreted in response to microbial insults and imparts a selective pressure for enterobactin-independent methods of iron acquisition for colonization of the urinary tract. In accordance with this hypothesis, we found that 70 of 80 of the UPEC strains isolated from women during episodes of cystitis expressed one or more of the siderophores aerobactin, salmochelin, or yersiniabactin. The association of higher siderophore numbers with higher urine *E. coli* levels is likely due to greater fitness of these strains in an iron-scarce environment. uLCN2 levels also tended to be higher during cystitis episodes caused by UPEC strains expressing more siderophore systems. A plausible explanation could be that UPEC strains expressing multiple siderophores induce a stronger inflammatory response in the urinary tract due to increased fitness and higher bacterial loads. Alternatively, higher LCN2 levels may be regulated by host genetic determinants and then the increased levels lead to selection of strains with multiple siderophores. Additional studies are needed to clarify these relationships.

LCN2 levels rapidly increased and decreased in mouse tissues and human urine around episodes of UTI and corresponded to organ and urine bacterial loads, respectively. A similar trend was seen for leucocyturia, which was expected because UPEC challenge will induce an inflammatory response, including induction of LCN2, and leukocyte recruitment. LCN2 is secreted by infiltrating neutrophils and also, as shown in this study, produced by mucosal epithelial cells. LCN2 thus seemed to mirror the onset and the resolution of infection, and also the infecting strain, as uLCN2 levels were higher in patients with UTI caused by Gram-negative bacteria than those with Gram-positive cultures. The sensitivity of uLCN2 to detect bacterial insults was further demonstrated, as uLCN2 levels were associated with bacteriuria levels during asymptomatic periods. uLCN2 is similarly shown to be increased in children with UTI (43), and a weak correlation between uLCN2 and bacterial counts has been found in a study of patients presenting with pyuria or UTI (44). However, to our knowledge, this is the first study to reveal a temporal association of uLCN2 levels with UTI episodes in individual women experiencing rUTI. In addition to a protective role, our results suggest that uLCN2 may be a biomarker for ASB, early detection of UTI, and treatment response.

Biological fluids contain low basal levels of LCN2 (~10–50 ng/ml in serum and urine) that is rapidly increased to high proportions (>1 μg/ml) in response to harmful insults. However, the cellular source of LCN2 in the various conditions is unclear, as LCN2 can be secreted from blood or tissue-infiltrating neutrophils, liver hepatocytes, mucosal epithelial cells, or resident cells of spleen and kidney (18, 20, 24, 32, 37, 45, 46). In our mouse study, LCN2 was increased in the urinary tract as early as 6 h after inoculation. Immunohistological examination revealed $LCN2^+$ neutrophils followed by induction of LCN2 in the urothelium of infected bladders, thus satisfying the biomarker principle of secretion from insulted cells. The induction of LCN2 in bladder epithelial cells could, however, be indirectly induced by IL-17a, IL-22 or IL-1β (24, 32, 35, 47, 48), cytokines that were highly induced in the bladder. We did not test the requirement of cytokines for LCN2 induction in mouse urothelium. However, we have found that a urothelial cell line induced secretion of LCN2 in response to IL-17a and IL-22, and not in response to direct UPEC challenge (T.H. Flo, unpublished observations). Barasch and colleagues (37) previously found that kidneys are the major source of uLCN2 during ischemic injury. To our knowledge, our study is the first to show that LCN2 is locally and sensitively produced in the bladder mucosa during UPEC challenge. Taken together with our finding that uLCN2 is elevated in patients with ASB, care should be taken to rule out bacteriuria when considering using uLCN2 as a biomarker for kidney injury.

A major strength of the present study is the prospective follow-up of a large cohort of women with good compliance in the daily collection of specimens over time and characterization of the inflammatory and microbial events prior to rUTI episodes. Still, there are limitations. Only 14 of the patients could be used for uLCN2 comparisons during the days preceding rUTI events. More specimens from more patients would be needed to yield valid statistical analyses of the full time course of uLCN2 levels related to cystitis episodes. Also, findings in the present study may not be identical for sporadic UTI, as women were enrolled based on a history of rUTI. We also did not measure enterobactin or other nonsiderophore iron acquisition systems, such as heme, in the patient-derived UPEC strains. The heme uptake system in LCN2-resistant strains may be an overwhelming factor related to LCN2 levels, and its absence in our panel of siderophores may unduly undermine the detection of association of LCN2 levels with the number of iron-acquiring siderophores (49). Finally, the clinical cohort used in the present study was not designed to address the most pressing question, that is, whether there is a risk for UTI associated with low basal LCN2 levels or LCN2 responsiveness (time and level of induction) of an individual. A large and prospective follow-up study of sporadic UTI could possibly provide some answers.

We show, to our knowledge, for the first time that LCN2 is rapidly and locally produced in the urinary tract in response to bacterial challenges and confers a selective pressure on the type of siderophores required by uropathogenic *E. coli* for establishing UTI. The sensitivity of uLCN2 to detect bacterial insults was further demonstrated, as uLCN2 levels were associated with bacteriuria levels during asymptomatic periods. Although elevated levels of LCN2 in kidneys and in the urine in response to damage or infection have been demonstrated, to our knowledge, the present study is the first to establish a protective role for LCN2 in UTI. Rapid and proportional secretion of LCN2 by urothelial cells all along the urinary tract in response to danger could be a protective measure in situations where the urinary tract is vulnerable to microbial insults or therapeutic during ongoing infections. In either case, our results describe new and important mechanisms for UTI pathogenesis and point to a putative role of LCN2 as a sensitive biomarker of microbial colonization and early detection of infection of the urinary tract.

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Disclosures

The authors have no financial conflicts of interest.

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