

RESEARCH ARTICLE

Expression and Significance of the HIP/PAP and RegIIIy Antimicrobial Peptides during Mammalian Urinary Tract Infection

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Abstract

Recent evidence indicates that antimicrobial peptides (AMPs) serve key roles in defending the urinary tract against invading uropathogens. To date, the individual contribution of AMPs to urinary tract host defense is not well defined. In this study, we identified *Regenerating islet-derived 3 gamma* (RegIIIy) as the most transcriptionally up-regulated AMP in murine bladder transcriptomes following uropathogenic *Escherichia coli* (UPEC) infection. We confirmed induction of RegIIIy mRNA during cystitis and pyelonephritis by quantitative RT-PCR. Immunoblotting demonstrates increased bladder and urinary RegIIIy protein levels following UPEC infection. Immunostaining localizes RegIIIy protein to urothelial cells of infected bladders and kidneys. Human patients with UTI have increased urine concentrations of the orthologous Hepatocarcinoma-Intestine-Pancreas / Pancreatitis Associated Protein (HIP/PAP) compared to healthy controls. Recombinant RegIIIy protein does not demonstrate bactericidal activity toward UPEC *in vitro*, but does kill *Staphylococcus saprophyticus* in a dose-dependent manner. Kidney and bladder tissue from RegIIIy knockout mice and wild-type mice contain comparable bacterial burden following UPEC and Gram-positive UTI. Our results demonstrate that RegIIIy and HIP/PAP expression is induced during human and murine UTI. However, their specific function in the urinary tract remains uncertain.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The human urinary tract ranks among the most common sites of bacterial infections.[1] Over 50% of women experience at least one case of urinary tract infection (UTI) in their lifetime.[2] While most UTI localize to the bladder (cystitis), ascending bacteria can invade the renal parenchyma, resulting in acute pyelonephritis (APN). In 2012, APN led to 200,000 emergency department visits, 64,000 hospital admissions, and \$378 million in inpatient hospital costs in the United States alone.[3] The frequency and severity of UTI increase in patients with urinary tract obstruction, cystic kidney disease, neurogenic bladder, kidney stones, diabetes mellitus, and recipients of kidney transplants.[4] Treatment for recurrent UTI exposes patients to repeated courses of antibiotics, which promotes the emergence of antibiotic-resistant pathogens and restricts the physician's treatment options.[5] Altogether, this mandates a greater understanding of UTI pathogenesis in order to thwart invading bacteria and protect the urinary tract.

The innate immune response is responsible for swift and efficient eradication of pathogens, as well as triggering the adaptive immune response. In the urinary tract, invading bacteria face defenses consisting of (1) bladder emptying and mucous production; (2) exfoliation and regeneration of the urothelial epithelium; (3) recruitment of phagocytes that possess bactericidal activity and engulf microbes; (4) cytokines and chemokines; (5) and AMPs.[6,7,8,9,10] Recent evidence suggests that AMPs protect the urothelium from invading pathogens. Urothelial cells or invading leukocytes produce AMPs, like α - and β -defensins, cathelicidin, and ribonuclease 6 and 7, that directly kill uropathogens. Other urinary tract proteins, including lipocalin and lactoferrin, suppress bacterial growth by scavenging key micronutrients.[6,7,8,9]

The goal of this study was to identify AMPs induced during experimental UTI using an unbiased global transcriptome profiling approach. In doing so, we identified *Regenerating islet-derived 3 gamma* (RegIII γ) as the most transcriptionally induced AMP in mouse bladders. RegIII γ , which was initially identified in Paneth cells of the small intestine, is a secreted lectin that exerts antimicrobial activity against Gram-positive bacteria. Prior studies implicate RegIII γ as a mediator of mucosal immunity in other organ systems. [11,12,13,14,15,16,17] Therefore, in this study we localized RegIII γ urinary tract expression and assessed its role in uropathogen clearance.

Methods

Bacterial strains

UTI89 is a type I-piliated UPEC strain isolated from a patient with cystitis.[18] CFT073 is a UPEC strain isolated from the blood and urine of a patient with APN.[19] We also used Gram-positive human urinary tract isolates *Enterococcus faecalis* 0852 and *Staphylococcus saprophyticus* 7108. [20] [21,22]

UTI model

Use of the RegIII γ ^{-/-} and WT mice for experimental UTI model was approved by The Research Institute at Nationwide Children's Hospital Institutional Laboratory Animal Care and Use Committee (Welfare Assurance Number A3544-01), protocol AR13-00057 (BB). Animals were euthanized by cervical dislocation under deep isoflurane anesthesia. Internal organs were removed to ensure demise. RegIII γ ^{-/-} mice were generously provided by Dr. Lora Hooper (UT Southwestern) and backcrossed at least 7 generations to C57BL/6 prior to experiments.[15] RegIII γ ^{-/-} or C57BL/6 (wild-type (WT)) females were generated by homozygous breeding and maintained on standard chow (Harlan, Indianapolis, IN) with 12 hour light/dark cycles.

Animals were housed in ventilated cages and transferred to a Biosafety Level 2 room one day prior to inoculation. Uropathogenic bacteria were inoculated from glycerol stocks into LB medium and expanded statically for 16 hours at 37°C. Experimental UTI, tissue isolation, and assessment of bacterial burden were performed as previously described.[23] The bacterial inoculum was 10⁸ colony forming units (CFU) in 50 μ l except where otherwise indicated.

RNA extraction and microarray hybridization

Total RNA was extracted using the mirVanaTM kit (Life Technologies, ThermoFisher Scientific). Tissue disruption was accomplished with a TissueLyser II instrument (Qiagen, Carlsbad, CA). Sample concentration was determined using the NanoDrop[®] ND-1000, and RNA integrity was analyzed using the Agilent 2100 Bioanalyzer Lab-On-A-Chip Agilent 6000 Series II chip. Sample labeling and hybridization was performed per the manufacturer's protocol. Samples were hybridized to the SurePrint G3 Mouse GE 8x60K Microarray and scanned using the Agilent G2505C Microarray Scanner. Raw data were quality-assessed, filtered for outliers and normalized to remove non-biological variation. A 10% false discovery rate and p-value adjustment using the Bioconductorlimma package yielded significantly differentially expressed genes (Core Facility, Nationwide Children's, Columbus, OH). Microarray data may be accessed at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/GEO), using accession number GSE72007.

QRT-PCR

Up to 2 μ g of total RNA were reverse transcribed using random hexamer oligonucleotides in a 20 μ l reaction volume (Verso cDNA Synthesis Kit, Thermo Scientific, Waltham, MA). After dilution to 60 μ l with sterile water, 1 μ l cDNA was used as template in a quantitative (q) RT-PCR reaction. Duplicate PCR reactions were performed using 2x master mix (Fisher). VIC-MGB labeled *Gapdh* and FAM-MGB labeled *RegIII γ* or *RegIII β* primer/probe sets were used in separate reactions (Applied Biosystems, Carlsbad, CA). Alternatively, PCR reactions were performed using 2x master mix containing Sybr Green (Fisher) and 1 pmol of gene-specific primer pairs (S1 Table). PCR products were amplified and detected using the 7500 Real-Time PCR System (Applied Biosystems). PCR threshold cycles (CT) were determined, and each cDNA was normalized for *Gapdh* content (Δ CT). Relative expression changes were calculated using the 2 ^{$-\Delta\Delta$ CT} method, normalizing to a common uninfected kidney or bladder cDNA pool.[24]

Immunoblotting and immunostaining

Western blot analysis and immunostaining were performed as previously described.[23,25] Primary antibody used for Western blot analysis included: rabbit anti-RegIII γ antibody, 1:1000, Dr. Lora Hooper, UT Southwestern, Dallas, TX; mouse anti-*Gapdh* antibody, MAB374, Chemicon, 1:5000. For immunofluorescence (IF), the RegIII γ antibody was diluted 1:400, then detected with AlexaFluor 488-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Negative controls sections were incubated with rabbit serum in place of RegIII γ antisera.

Study approval and human urine specimens

Informed written consent was obtained from all patients participating in this study. For subjects less than 18 year of age, written parental/guardian consent was obtained. The Nationwide Children's Hospital (NCH) Institutional Review Board approved this study along with the consent process and documents (IRB07-00383).[26] Non-infected and infected urines samples

were obtained from children presenting to the NCH emergency department or the nephrology clinic. The diagnosis of a UTI was made by a positive urine culture according to the American Academy of Pediatrics Guidelines.[27] All infected urine samples had $>10^5$ CFU/mL *E. coli*.

Human urinary HIP/PAP levels

Urinary HIP/PAP (PAP) concentrations were measured by enzyme linked immunosorbent assay (ELISA; Dynabio, Marseille, France). Results were normalized to urine creatinine (Cr) as measured by ELISA (Oxford Biomedical Research, Rochester Hills, MI).

Amino acid alignments: Full-length Human HIP/PAP (accession number NP_620355.1) and mouse RegIII γ (NP_035390.1) amino acid sequences were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/protein) and aligned using MegAlign (DNASStar, Madison, WI) with the Clustal W method.

Bactericidal activity of recombinant RegIII γ

Recombinant, N-terminally processed RegIII γ was purified from *E. coli* inclusion bodies as described.[28,29] UPEC strain UTI89 (10^5 CFU) was incubated with 0–10 μ M RegIII γ for 2 hours at 37°C in 10 mM MES, pH 5.5 with 25 mM NaCl. Samples were then serially diluted, plated overnight on LB, and colonies were enumerated.

Statistics

For comparing bacterial burden in WT versus RegIII γ ^{-/-} mice, geometric means of log-transformed CFUs from kidneys and bladders were compared by the Mann-Whitney U test (Graph-Pad Software, La Jolla, CA). Similarly, for comparison of fold-differences in mRNA expression, Mann-Whitney U test was used. *P* values of < 0.05 were considered significant.

Results

Global analysis of AMP mRNA expression in experimental cystitis

To identify AMP undergoing transcriptional up-regulation by UPEC in an unbiased manner, we evaluated bladder transcriptomes at baseline and following experimental UTI by oligonucleotide microarray. We performed experimental cystitis in C57BL/6J female mice, and compared transcriptomes from uninfected bladders versus bladders harvested 2, 6, and 48 hours following intravesical inoculation with 10^8 CFU of UPEC strain UTI89. We interrogated these data using 98 *Mus musculus* gene symbols encoding known or putative AMPs (S2 Table [6,30,31]) and identified 7 AMP transcripts with ≥ 4 -fold increase in expression during experimental cystitis (Table 1). These genes encode AMPs with varying mechanisms of antimicrobial activity including bactericidal activity (*RNase6*, *RegIII γ*) as well as metal sequestration (*S100a8*, *S100a9*, *Lcn2*, *Ltf*).[6,13]

Previously, we demonstrated that significant *RNase6* mRNA induction occurs 48 hours post infection (hpi) with UPEC.[23] Therefore, we evaluated expression of the remaining 6 AMPs induced during UTI by quantitative reverse transcription polymerase chain reaction (qRT-PCR) at 6 and 24 hpi (Fig 1). QRT-PCR confirmed significant induction of *RegIII β* , *RegIII γ* , *Lcn2*, *Ltf*, *S100a8*, and *S100a9* mRNA levels following infection (Fig 1). Our results also identify *RegIII γ* as the AMP with the highest fold-induction following UPEC challenge.

RegIII γ mRNA and protein expression during cystitis

In non-infected urinary tract tissues, qRT-PCR demonstrates significantly higher expression of *RegIII γ* mRNA in bladder compared to ureter and kidney ($p = 0.0286$, Mann-Whitney U test)

Table 1. Regulation of AMP mRNA expression during experimental cystitis. Genes encoding known or putative AMP with ≥ 4-fold change (FC) are depicted. False discovery rate (FDR) < 10 is considered significant; n.s. = not significant.

AMP	2 HPI		6 HPI		48 HPI	
	FC	FDR	FC	FDR	FC	FDR
<i>Reg3γ</i>	15.5	2.2	167.4	1.3	90.1	1.7
<i>S100a9</i>	5.3	4.1	59.4	1.3	14.5	7.6
<i>S100a8</i>	4.0	3.3	39.3	1.3	10.4	7.3
<i>Lcn2</i>	n.s.	n.s.	9.1	1.3	4.9	8.7
<i>Ltf</i>	n.s.	n.s.	5.1	1.3	12.5	3.2
<i>Reg3β</i>	n.s.	n.s.	4.3	1.3	n.s.	n.s.
<i>RNase6</i>	n.s.	n.s.	n.s.	n.s.	4.3	2.1

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(S1 Fig). To completely characterize *RegIIIγ* mRNA expression during experimental UTI, we profiled *RegIIIγ* mRNA expression over a more extensive time course. As predicted by our microarray experiment, *RegIIIγ* mRNA levels significantly increase as early as 2 hpi and peak 16 hpi with 300-fold higher expression over uninfected bladders ($p = 0.0286$, Mann-Whitney U test)(Fig 2A). The induction of *RegIIIγ* mRNA was specific to UPEC, as intravesical injection of PBS carrier or inoculation of the Gram-positive uropathogens, *Enterococcus faecalis* and *Staphylococcus saprophyticus*, did not elicit a significant expression change (Fig 2B).

To determine if RegIIIγ protein expression parallels *RegIIIγ* mRNA expression, we performed Western blot analysis. Following UPEC inoculation, we detected RegIIIγ protein by 16 hpi (Fig 2C) and as late as 72 hpi (data not shown). In addition, we identified RegIIIγ protein in urine of mice 24 hpi with UPEC, but not in sterile urine or following intravesical administration of Gram-positive uropathogens (Fig 2D). In urine, we visualized a more rapidly migrating RegIIIγ-immunoreactive band, consistent with reports that secreted RegIIIγ undergoes proteolytic processing.[12,29] RegIIIγ protein was not detected in *RegIIIγ*^{-/-} bladders or urine following UPEC inoculation (Fig 2C and 2D).

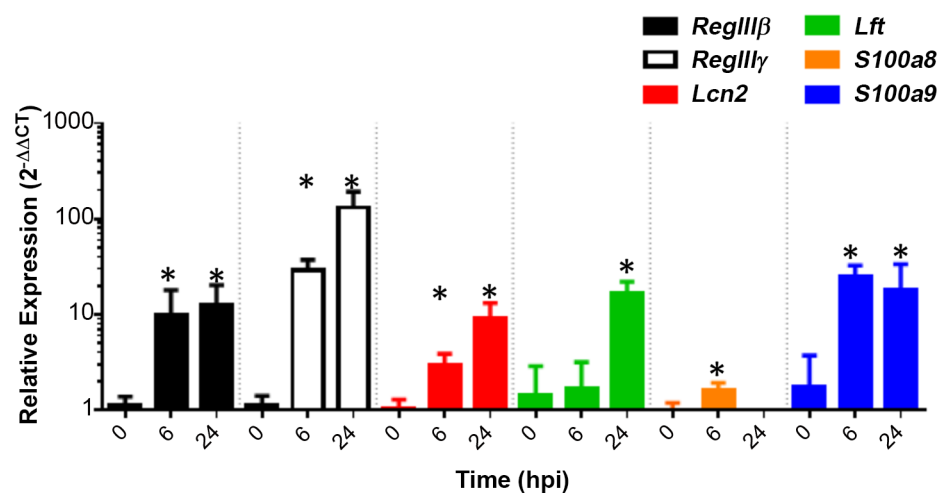


Fig 1. Confirmation of AMP mRNA regulation during experimental cystitis by qRT-PCR. The fold-change in AMP mRNA expression 6 and 24 hpi, relative to a pool of uninfected bladders, is shown. * $P < 0.05$, Mann-Whitney U test versus uninfected bladders (n = 4 bladders/group, uninfected and 6 hpi; n = 5 bladders/group, 24 hpi).

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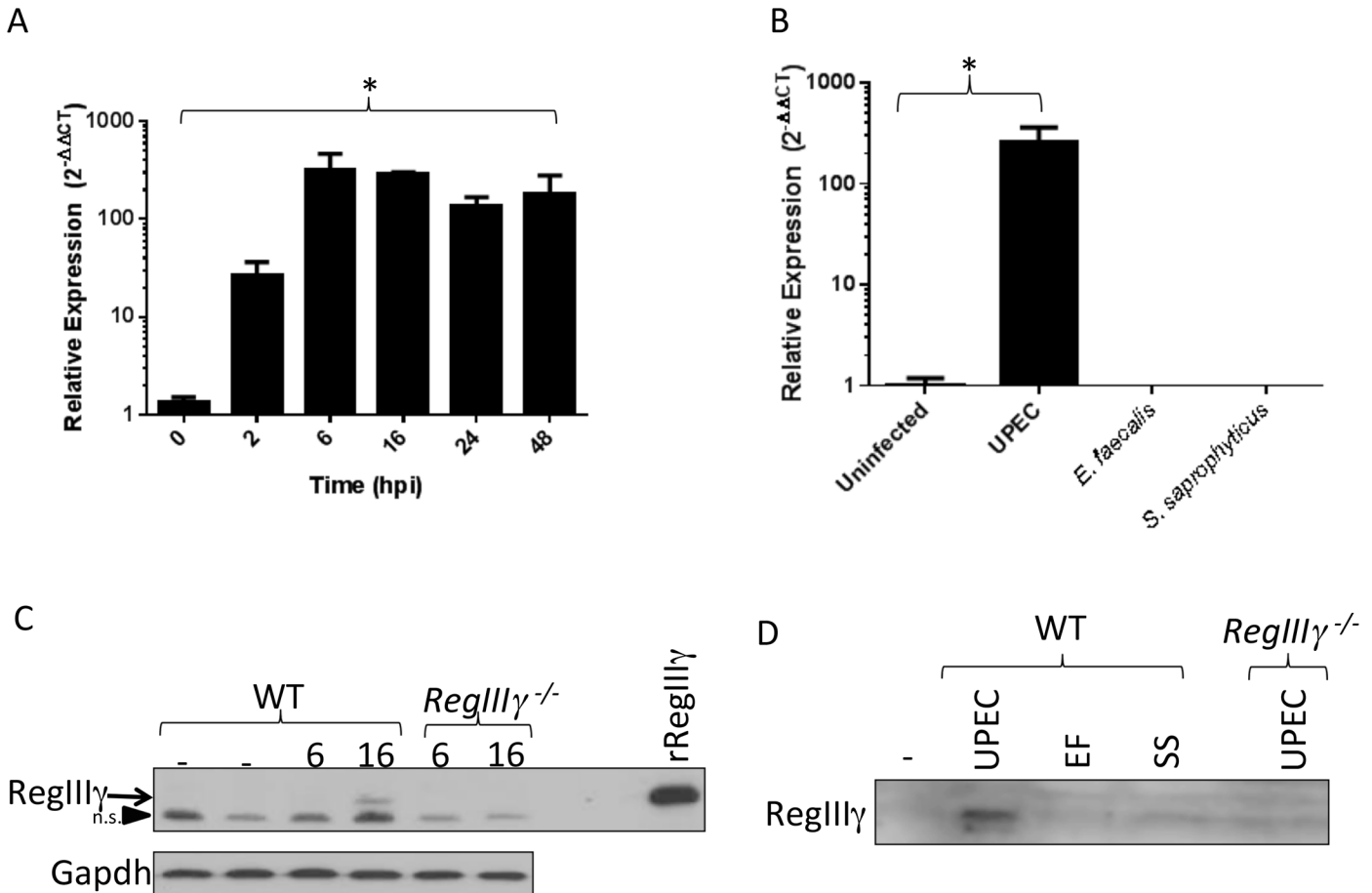


Fig 2. *RegIIIγ* mRNA and *RegIIIγ* protein expression are induced during UPEC cystitis, and *RegIIIγ* is secreted into the urinary stream following UPEC infection. (A) Fold-change in bladder *RegIIIγ* mRNA levels in response to intravesical inoculation of 10^8 CFU of UPEC strain UTI89. * $P = 0.0286$, Mann-Whitney U test versus uninfected bladders ($n = 4$ bladders/group). (B) Relative *RegIIIγ* mRNA levels increase 24 hpi following inoculation with 10^8 CFU of UPEC, but not in response to Gram-positive uropathogens. * $P = 0.0286$, Mann-Whitney U test, UPEC versus uninfected bladders ($n = 4$ bladders/group). (C) *RegIIIγ* protein (arrow) is induced in wild-type (WT) bladder 16 hpi with UPEC, but expression is absent at earlier times, and as well as in *RegIIIγ*^{-/-} bladders. Bladder *RegIIIγ* co-migrates with recombinant (r) *RegIIIγ* protein. A non-specific reactive band (n.s., arrowhead) migrates more rapidly and is present in WT and *RegIIIγ*^{-/-} bladder lysates. (D) *RegIIIγ* protein is undetectable in sterile WT urine, but secreted 24 hpi with UPEC. Gram-positive inocula, *E. faecalis* (EF) and *S. saprophyticus* (SS), fail to induce *RegIIIγ* secretion, and *RegIIIγ* immunoreactivity is absent in *RegIIIγ*^{-/-} urine 24 hpi with UPEC.

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To localize *RegIIIγ* expression in bladders following UPEC infection, we performed immunostaining. *RegIIIγ* was undetectable in naïve bladders and at 2 and 6 hpi. Beginning 16 hpi we observed strong, patchy reactivity within urothelium by immunohistochemistry (Fig 3). Immunofluorescence microscopy confirmed cytoplasmic localization of *RegIIIγ* within urothelial cells, along with apical enrichment of *RegIIIγ* expression (S2 Fig). *RegIIIγ* immunoreactivity intensified 24 hpi, and remained detectable up to 72 hpi (Fig 3 and S2 Fig).

RegIIIγ mRNA and protein expression during pyelonephritis

To evaluate the expression of *RegIIIγ* during pyelonephritis, we challenged C3H/HeOuJ mice with UPEC (strain CFT073). The C3H/HeOuJ strain is more susceptible to pyelonephritis following transurethral UPEC inoculation.[32,33,34] When challenged with 10^8 CFU UPEC, C3H/HeOuJ kidneys developed persistent bacterial burden up to 28 days post infection (dpi;

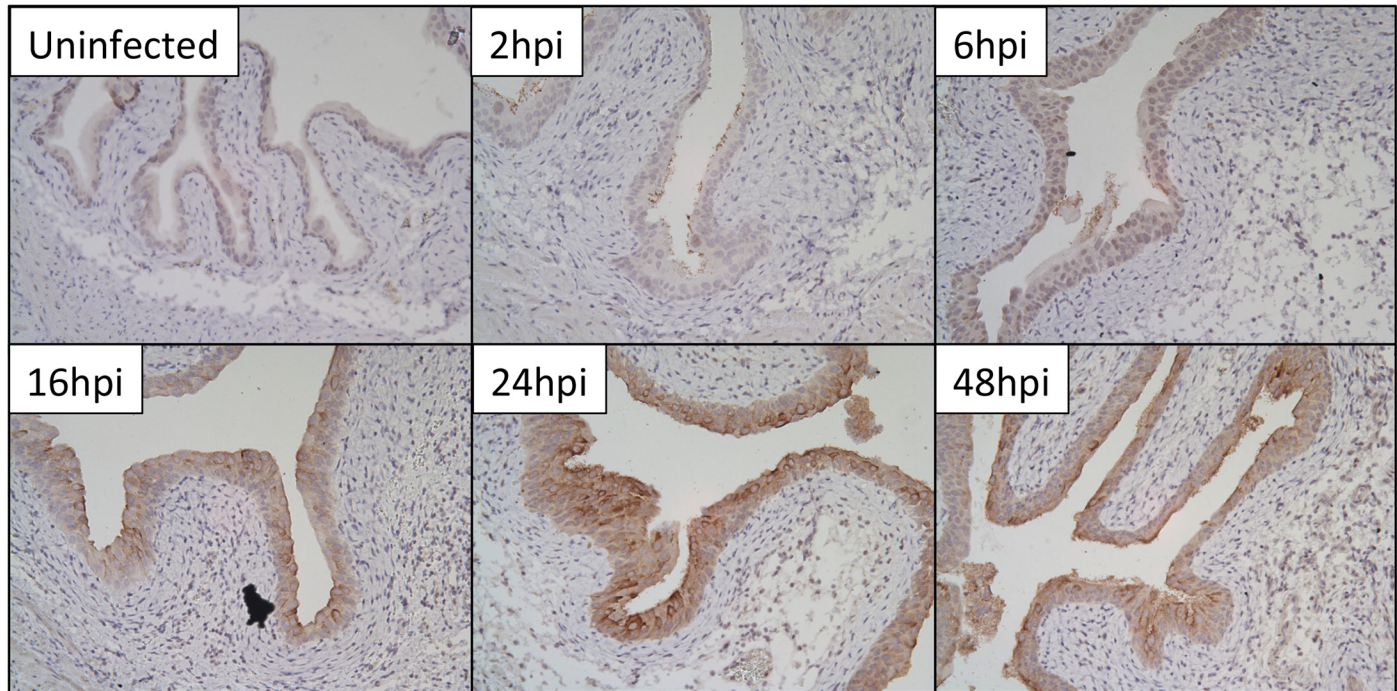


Fig 3. RegIII γ protein localizes to urothelium following UPEC infection. RegIII γ immunoreactivity is absent in uninfected bladders. Non-specific staining of UPEC is seen 2 and 6 hpi. Beginning 16 hpi, patchy urothelial immunoreactivity is observed. Urothelial RegIII γ expression is most prominent 24 hpi and persists to a lesser extent 48 hpi. All figures are 20x magnification.

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S3 Fig. Renal RegIII γ mRNA levels significantly increase 1 dpi and continue to rise at later time points (14 and 28 dpi; **Fig 4A**). Uninfected C3H/HeOuJ renal urothelium exists as a monolayer with uniformly apical Uroplakin 3a (Upk3a) and absent RegIII γ protein expression (**Fig 4B**). At 28 dpi, the renal urothelium appeared hyperplastic with reduced Upk3a expression and diffuse expression of RegIII γ protein (**Fig 4C**).

Urinary HIP/PAP levels increase during UTI

In humans, the Hepatocarcinoma-Intestine-Pancreas / Pancreatitis Associated Protein (HIP/PAP) is orthologous to RegIII γ and 67% identical at the amino acid level (**S4 Fig**). ELISA analysis demonstrates that human urinary HIP/PAP concentrations significantly increase during *E. coli* UTI (**Fig 5A**). 26 patients with positive urine cultures had average urine PAP/Cr ratios of 161.2 pg/mg (95% confidence interval [CI], 77–245 pg/mg), compared to 26 culture-negative urines that exhibited PAP/Cr ratios of 17.05 (95% CI, 5–29 pg/mg); $p < 0.0001$, Mann-Whitney U test. Western blots also demonstrated higher HIP/PAP levels in infected urine (**Fig 5B**).

Recombinant RegIII γ protein does not kill UPEC in vitro, and RegIII γ deficiency does not affect UTI susceptibility in vivo

To determine whether RegIII γ displays bactericidal activity toward uropathogenic bacteria, we expressed recombinant (r)RegIII γ protein in *E. coli* and purified it from bacterial inclusion bodies. [12,28,29] Antimicrobial kill assays show that rRegIII γ kills *S. saprophyticus* in dose-dependent fashion, but does not exhibit bactericidal activity against UPEC (strain UTI89, **Fig 6A**).

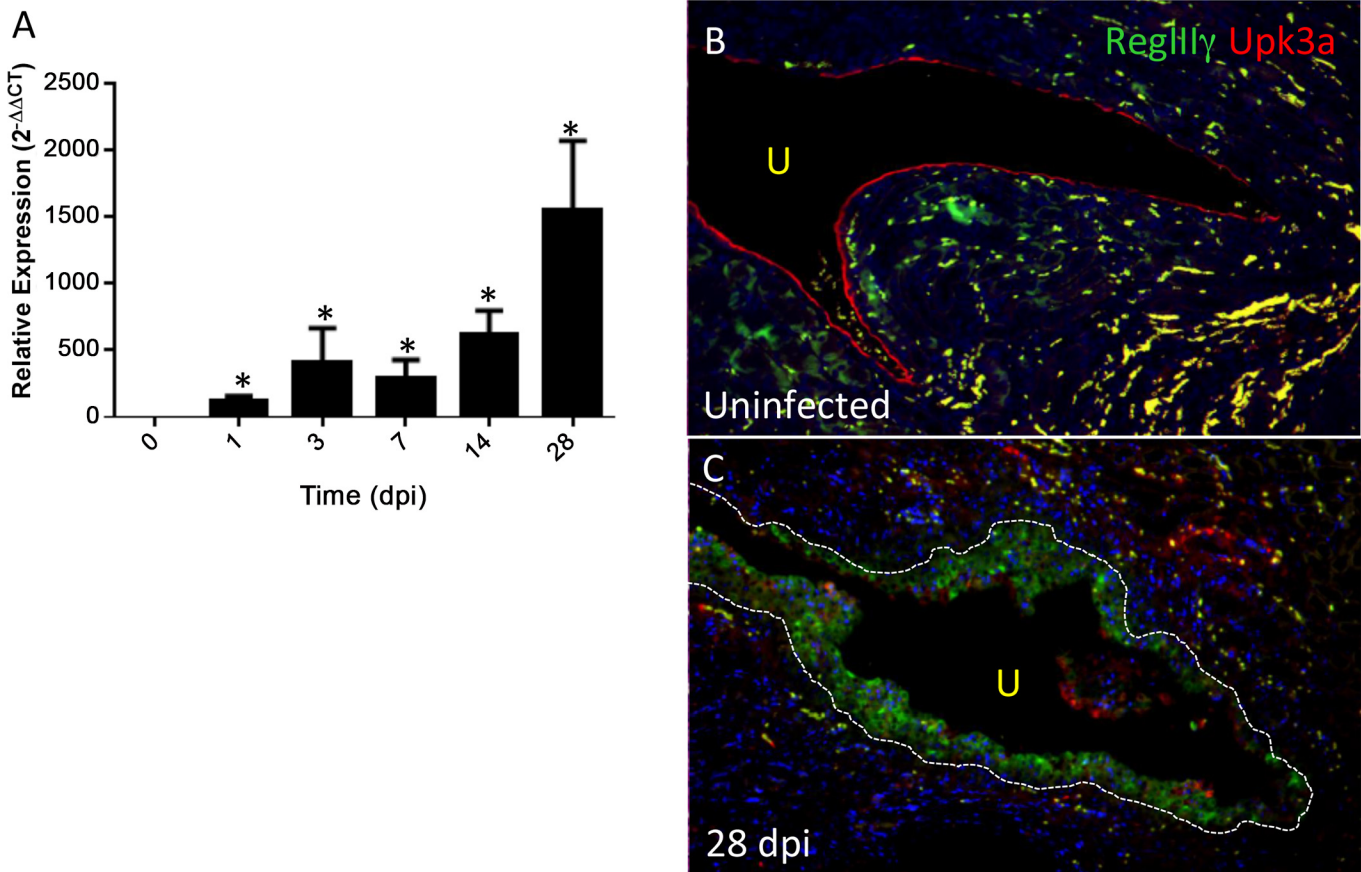


Fig 4. Induction of *RegIIIγ* mRNA and *RegIIIγ* protein by UPEC in C3H/HeOuJ kidneys. (A) Significant *RegIIIγ* mRNA induction by UPEC in C3H/HeOuJ kidneys, with progressive accumulation over time. (* $P = 0.0286$, Mann-Whitney U test versus uninfected C3H/HeOuJ kidneys, $n = 4$ kidneys/time). (B) Absent *RegIIIγ* immunostaining in uninfected C3H/HeOuJ urothelium, which exhibits uniform apical expression of Uroplakin 3a (Upk3a; red). U: Urinary space. (C) *RegIIIγ* protein (green) localizes to the hypertrophied urothelium of C3H/HeOuJ kidneys 28 dpi, with reduced expression of Upk3a. Urothelial border is indicated by dashed line. Both images are 20x magnification.

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To confirm these results in a model system, we evaluated the effects of *RegIIIγ* deletion on bacterial clearance. We confirmed absence of *RegIIIγ* mRNA and *RegIIIγ* protein in *RegIIIγ*^{-/-} bladders by qRT-PCR, immunostaining, and immunoblotting following experimental UTI (Fig 2C, S2 Fig, and data not shown). We used an inoculum of 10⁸ CFU of UPEC strain UTI89, identical conditions to those in which we originally identified *RegIIIγ* mRNA expression, and investigated bacterial burden 24 hpi. We did not observe significant differences in urine, bladder, ureter, or kidney bacterial burden between *RegIIIγ*^{-/-} and wildtype C57BL/6J control mice (Fig 6B). We also did not observe a difference in bacterial burden when the inoculum was reduced to 10⁶ or 10⁷ CFU, or when we evaluated bacterial burden 16, 24, 48, or 72 hpi (S5 Fig). Since *RegIIIγ* preferentially kills Gram-positive bacteria, we performed parallel experiments challenging *RegIIIγ*^{-/-} and wild-type mice with 10⁸ CFU of uropathogenic *Staphylococcus saprophyticus* and *Enterococcus faecalis*. However, we did not observe any difference in susceptibility of *RegIIIγ*^{-/-} mice to these Gram-positive bacteria 24 hpi (Fig 6C and S6 Fig).

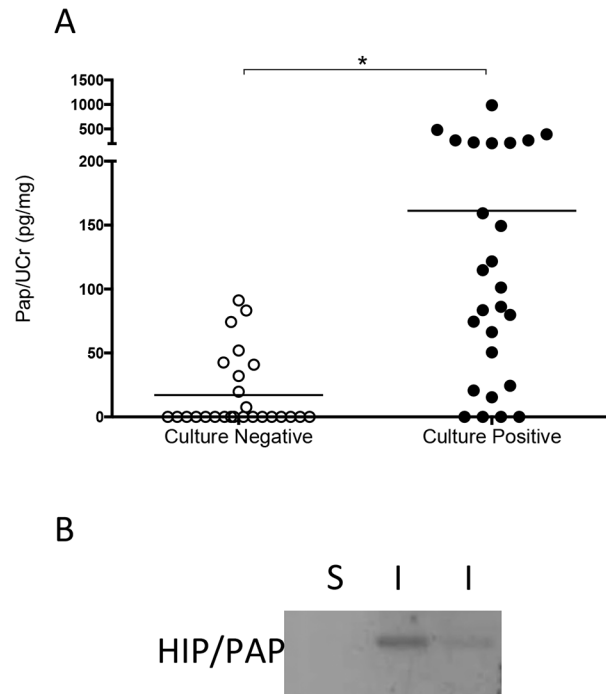


Fig 5. The human RegIIIγ orthologue, HIP/PAP, is secreted in infected human urine. (A) HIP/PAP levels were quantified by ELISA and adjusted for urine creatinine to control for differences in urine concentration. The resulting PAP/Cr ratio is significantly elevated in urine from patients with positive urine cultures, compared to sterile urine (n = 26 urines/group; * $P < 0.0001$, Mann-Whitney U test). The horizontal line indicates the mean. (B) Western blotting demonstrates absent of HIP/PAP in sterile (S) urine, whereas infected (I) urine from two children with UTI exhibits immunoreactivity.

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Discussion

Bladder AMPs are identified on the basis of their transcriptional induction by UPEC

The purpose of this study was to identify AMPs within the bladder on the basis of their transcriptional induction during experimental UTI. This unbiased approach identified a set of AMPs with diverse mechanisms of antimicrobial activity. Most of these AMPs have been evaluated in the setting of UTI. Loss of function studies have established a role for Lcn2 production by bladder urothelium and α -intercalated cells of renal collecting ducts in response to UPEC, achieved in part through iron scavenging.[35,36] Peptide fragments from human lactoferrin exhibit bactericidal activity toward UPEC *in vitro* and when administered orally accumulate in urine and reduce bladder and kidney colonization by UPEC *in vivo*.[37] S100a8 and S100a9 heterodimerize to form calprotectin, a neutrophil derived AMP with bacteriostatic activity due to chelation of micronutrient metals such as zinc and manganese.[38] Previous studies have demonstrated accumulation of neutrophil-derived S100a8 and S100a9 proteins during experimental UTI, but *S100a9* deletion does not confer increased susceptibility to infection.[39] Phagocytes express RNase6 protein during human and murine UTI, and recombinant human and murine RNase6 proteins are bactericidal toward UPEC *in vitro*.[23] Along with these AMPs, here we identified *RegIIIβ* and *RegIIIγ* as inducible AMPs during experimental UTI. This project focused on *RegIIIγ* due to its high magnitude of induction.

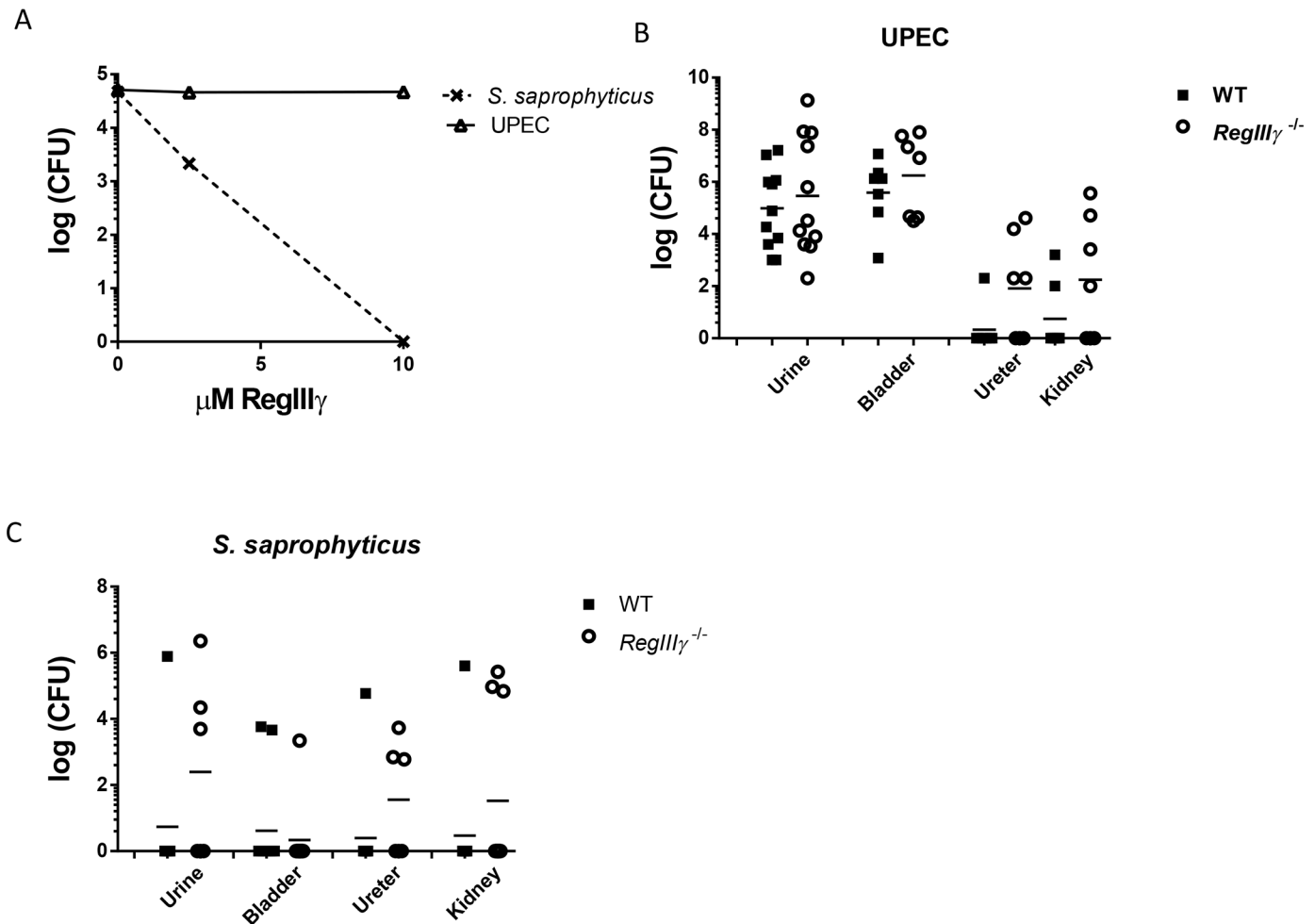


Fig 6. Recombinant RegIII γ protein exhibits bactericidal activity toward *S. saprophyticus* but not UPEC, and RegIII γ deletion does not affect susceptibility to experimental UTI. (A) Recombinant RegIII γ kills *S. saprophyticus* not UPEC in a dose-dependent manner. 10^5 CFU bacteria were incubated with indicated concentrations of recombinant C-terminally cleaved RegIII γ for 2 hours at 37°C in 10 mM MES, pH 5.5 with 25 mM NaCl, and colonies were enumerated. (B-C) Experimental UTI. Comparable bacterial burden in wildtype (WT) and RegIII γ ^{-/-} urinary tracts following inoculation with 10^8 CFU of UPEC strain UT189 (B) or *S. saprophyticus* (C). There was no significant difference between genotypes by Mann-Whitney U test ($p > 0.05$). Horizontal lines indicate geometric means.

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RegIII γ protein expression increases during UTI and localizes to urothelial cells

We confirmed induction of RegIII γ protein in infected mouse urine and bladders by immunoblotting and localized RegIII γ protein to infected bladder urothelium. Our study adds urothelium to the list of mucosal surfaces capable of RegIII γ expression. Previous studies have identified constitutive RegIII γ mRNA expression in intestinal epithelium and localized RegIII γ protein to mucus coating these cells.[14,15] In addition to this “homeostatic” pool of RegIII γ , mucosal injury leads to cytokine-mediated induction of RegIII γ production.[40,41] RegIII γ mRNA expression also occurs in developing airway epithelium as well as in lung epithelium during *S. aureus* pneumonia.[12,42,43] We were unable to detect RegIII γ protein in bladder extracts or localize RegIII γ protein to bladder urothelium in the absence of infection, but RegIII γ mRNA was readily detected under these conditions with higher levels in bladder than

other urinary tract organs. This suggests separate constitutive and inducible pools of RegIII γ mRNA, which are likely subject to differential regulation.

Potential mechanisms of RegIII γ mRNA regulation during UTI

While the mechanisms responsible for urothelial expression of RegIII γ mRNA are not elucidated in our study, certain inferences can be made based on published findings outside of the urinary tract. Homeostatic RegIII γ mRNA expression in intestine depends on Toll-like receptor (Tlr) 4 signaling in epithelial cells through the adaptor proteins Myd88 and Trif. [15,17,41,44] Intestinal RegIII γ mRNA expression is significantly impaired in germ-free mice, and bacterial colonization or administration of bacterial products such as lipopolysaccharide (LPS) or flagellin induces RegIII γ expression via Tlr4 and Tlr5, respectively. [13,16,45] These studies raise the question of whether RegIII γ induction by UPEC is dependent on LPS, flagellin, or a different bacterial product in the urinary tract.

Previous transcriptome studies have identified RegIII γ mRNA induction during cystitis and offer clues about its regulation by bacteria. [46,47,48,49] UPEC lacking the FimH adhesin elicit 9.5-fold less RegIII γ mRNA induction than the parental strain, attesting to a potential role in FimH interactions with uroplakin plaque covering the urothelium in RegIII γ expression. [47,50] However, RegIII γ mRNA expression does not uniquely depend on Gram-negative bacteria, since intravesical inoculation of *Streptococcus agalactiae* results in 8.7-fold higher bladder RegIII γ mRNA levels versus uninfected controls. [46] Thus, multiple pathogen associated molecular patterns (PAMPs) may be responsible for bladder RegIII γ expression, just as LPS and flagellin regulate RegIII γ mRNA levels in the gut. [16,45]

Alternatively, induction of RegIII γ mRNA in UPEC infected bladders may occur as consequence of cytokine signaling. In the lung, interleukin-6 (IL-6) leads to phosphorylation of Signal transducer and activator of transcription (Stat) 3, which translocates to the nucleus and mediates RegIII γ mRNA transcription. [12,51] In the skin of mice and humans with psoriasis, HIP/PAP and RegIII γ undergo robust mRNA and protein induction under the direction of IL-17, another cytokine capable of eliciting Stat3 phosphorylation. [11] In the stomach, the *Helicobacter pylori* CagA protein elicits IL-11 production, which leads to Stat3 phosphorylation and RegIII γ expression. [52] In the mouse colon, infectious and chemical agents trigger production of IL-22, which induces epithelial RegIII γ expression. [40,53,54] Altogether, these observations warrant future studies of Stat3 and its upstream regulatory cytokines in the regulation of RegIII γ during experimental UTI.

Urinary RegIII γ and HIP/PAP are markers of urothelial injury

In this study, we demonstrate that RegIII γ and HIP/PAP proteins accumulate in the urine of mice and humans with UTI. Previous studies in rats and humans have identified induction of RegIII γ orthologues in the context of hemorrhagic cystitis, bladder cancer, and interstitial cystitis. [55,56,57] In rats, bladder *PAP-III* mRNA levels increase following cyclophosphamide treatment, and PAP-III protein uniquely localizes to the umbrella cells of bladders with hemorrhagic cystitis. [57] In humans with bladder cancer, HIP/PAP is detected in tumors by immunoblotting and urine HIP/PAP levels rise. [56] Patients with bladder cancer and IC exhibit elevated urine concentrations of HIP/PAP protein. [55,56] Taken together, these studies implicate RegIII γ and HIP/PAP as markers of urothelial injury. It will be insightful to determine the clinical and diagnostic value of urine HIP/PAP levels in patients with urothelial injury, including UTI and obstructive uropathy.

RegIII γ deletion does not confer susceptibility to experimental UTI

It is perhaps not surprising that genetic ablation of RegIII γ did not affect host susceptibility to UPEC UTI, since recombinant RegIII γ did not exert direct bactericidal activity toward UPEC. The antimicrobial activity of both RegIII γ and HIP/PAP requires binding to peptidoglycan, which is inaccessible in the Gram-negative cell wall.[13] Moreover, LPS inhibits multimerization of HIP/PAP and presumably RegIII γ , a required step for their membrane insertion into Gram-positive target cells.[58] Nonetheless, there is experimental precedent that RegIII γ promotes resolution of Gram-negative infections *in vivo*. Recombinant RegIII γ and HIP/PAP proteins prevent lethal *Citrobacter colitis*. [40] Moreover, genetic deletion of RegIII γ increases susceptibility to intestinal infection by *Salmonella enteritidis*, a Gram-negative bacterium.[14] Thus, while it is unlikely that RegIII γ or HIP/PAP exhibit direct microbicidal activity, it remains conceivable that they may work cooperatively with other AMPs or host immune cells to serve an antimicrobial function in certain Gram-negative infections.

Potential roles for RegIII γ and HIP/PAP in the urinary tract

While our findings argue against a direct antimicrobial role for urothelial RegIII γ during UTI caused by UPEC, alternative roles for RegIII γ merit consideration. In skin, RegIII γ and HIP/PAP promote keratinocyte proliferation and prevent terminal differentiation in states of wound repair and psoriasis.[11] Bladder urothelial cells exfoliate following UPEC exposure, prompting a wave of progenitor cell proliferation and differentiation that regenerates the mucosal layer.[59,60] Based on its function in skin and expression in urothelium, RegIII γ may regulate urothelial cell proliferation and/or differentiation during UTI. Alternatively, local expression of RegIII γ within the urothelium may serve immunomodulatory roles. In rats, the RegIII family member, PAP-I, promotes Stat3 phosphorylation in macrophages, blocking NF- κ B activation and inhibiting the inflammatory response.[61] This raises the question of whether RegIII γ ^{-/-} mice exhibit alterations in Stat3 activation and increased inflammation following experimental UTI. In support of this concept, bacteria directly interact with the epithelial surface in RegIII γ ^{-/-} intestine, leading to immune cell activation.[14,15]

Conclusions

UPEC infection leads to induction of RegIII γ mRNA and RegIII γ protein, which localizes to bladder and renal urothelial cells and is secreted in infected urine. RegIII γ and HIP/PAP represent novel biomarkers of human and murine UTI, respectively. The biological significance of these proteins in the infected urinary tract remains unknown.

Supporting Information

S1 Fig. RegIII γ mRNA levels are significantly higher in uninfected bladder, compared to ureter and kidney (* $P = 0.0286$, Mann-Whitney U test, $n = 4$ organs/group).

(TIF)

S2 Fig. RegIII γ expression by bladder urothelial cells following UPEC inoculation. Immunofluorescence microscopy localizes RegIII γ (green) to apical projections of wild-type (WT) urothelial cell layers 24 and 72 hpi. Simultaneously stained RegIII γ ^{-/-} sections exhibit non-specific staining at these times post infection. Nuclei are identified with DAPI (blue). Dashed lines demarcate the urothelial border. U: Urinary space. All images are 40x magnification.

(TIF)

S3 Fig. Persistent UPEC colonization of C3H/HeOuj kidneys. Female C3H/HeOuj mice with transurethrally inoculated with UPEC strain CFT073 (10^8 CFU). Dpi: days post infection. Squares indicate individual kidneys. The horizontal line indicates the geometric mean. (TIF)

S4 Fig. Human HIP/PAP is 67% identical in amino acid sequence to mouse RegIII γ . Identical amino acid residues are highlighted in black. (TIF)

S5 Fig. Effect of varying infection duration and magnitude of UPEC inoculum on urinary tract bacterial burden in RegIII γ ^{-/-} versus WT mice. (A) No significant difference in bladder or kidney bacterial burden was observed when WT and RegIII γ ^{-/-} mice were challenged with 10^7 CFU of UTI89 and sacrificed at 16, 24, 48, or 72 hpi ($p > 0.05$, Mann-Whitney U test). (B) No significant difference in urinary tract bacterial burden was noted when WT and RegIII γ ^{-/-} mice were challenged with 10^6 CFU of UTI89 and sacrificed 24 hpi ($p > 0.05$, Mann-Whitney U test). (TIF)

S6 Fig. Comparable bacterial burden in wildtype (WT) and RegIII γ ^{-/-} urinary tracts following inoculation with 10^8 CFU of *E. faecalis* strain 0852. There was no significant difference between genotypes by Mann-Whitney U test ($p > 0.05$). Horizontal lines indicate geometric means. (TIF)

S1 Table. QRT-PCR primers.
(XLSX)

S2 Table. *Mus musculus* genes encoding known or putative antimicrobial peptides [6,27,28].
(XLSX)

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Author Contributions

Conceived and designed the experiments: JDS ALS KMM BB. Performed the experiments: JDS ARJ BL CBC MV RSE BB. Analyzed the data: JDS ARJ BL RSE BB. Wrote the paper: JDS BB.

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