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Interleukin-6/Stat3 signaling has an essential role in the host antimicrobial response to urinary tract infection

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Abstract

The signaling networks regulating antimicrobial activity during urinary tract infection (UTI) are incompletely understood. Interleukin-6 (IL-6) levels increase with UTI severity, but the specific contributions of IL-6 to host immunity against bacterial uropathogens are unknown. To clarify this we tested whether IL-6 activates the Stat3 transcription factor, to drive a program of antimicrobial peptide gene expression in infected urothelium during UTI. Transurethral inoculation of uropathogenic *Escherichia coli* led to IL-6 secretion, urothelial Stat3 phosphorylation, and activation of antimicrobial peptide transcription, in a Toll-like receptor 4-dependent manner in a murine model of cystitis. Recombinant IL-6 elicited Stat3 phosphorylation in primary urothelial cells *in vitro*, and systemic IL-6 administration promoted urothelial Stat3 phosphorylation and antimicrobial peptide expression *in vivo*. IL-6 deficiency led to decreased urothelial Stat3 phosphorylation and antimicrobial peptide mRNA expression following UTI, a finding mirrored by conditional *Stat3* deletion. Deficiency in *IL-6* or *Stat3* was associated with increased formation of intracellular bacterial communities, and exogenous IL-6 reversed this phenotype in *IL-6* knockout mice. Moreover, chronic IL-6 depletion led to increased renal bacterial burden and severe pyelonephritis in C3H/HeOJ mice. Thus, IL-6/Stat3 signaling drives a transcriptional program of antimicrobial gene expression in infected urothelium, with key roles in limiting epithelial invasion and ascending infection.

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Keywords

urinary tract infection; antimicrobial peptide; IL-6; Stat3; urothelium; intracellular bacterial community

Introduction

Urinary tract infections (UTI) represent a modern public health crisis, particularly in light of UTI prevalence, widespread prescription of broad-spectrum antibiotics, and rising antimicrobial resistance among bacterial uropathogens.^{1–3} To care for patients with UTI, alternative treatments and prophylactic measures must be developed.^{4–6} The urothelium serves as the front line of urinary tract defense against invading microorganisms, with key roles in barrier function, pathogen detection, production of antimicrobial substances, and phagocyte recruitment.^{7, 8} Augmenting the innate immune properties of urothelium represents an attractive approach to UTI prevention and treatment.

The urothelium secretes and responds to chemokines and cytokines as an important component of its response to UTI.⁹ For example, urothelial interleukin (IL)-8 production and receptor expression serve critical roles in neutrophil recruitment.^{10–12} Likewise, IL-6 is rapidly secreted by urothelial cells following *Escherichia coli* exposure.^{13–15} Studies have implicated bacterial determinants, such as lipopolysaccharide (Lps) and P-fimbriae, in eliciting urothelial IL-6 production.^{16–18} In bladder urothelium, Lps engages the Toll-like receptor (Tlr) 4, triggering an intracellular signaling cascade that leads to IL-6 secretion.^{13, 15, 18}

Studies suggest IL-6 secretion carries functional significance during UTI. Uropathogenic *E. coli* (UPEC) strains – unlike laboratory *E. coli* strains – suppress IL-6 secretion by urothelial cells *in vitro*, and this is a proposed escape mechanism for UPEC to evade the host immune response.^{19, 20} UPEC virulence correlates inversely with IL-6 production, such that low IL-6 elicitation confers a survival advantage for UPEC during cystitis.^{19, 21, 22} IL-6 expression is also indicative of clinical UTI severity: children with pyelonephritis and renal scarring have elevated urine and serum IL-6 levels compared to those with cystitis.^{23–26} Animal models similarly demonstrate an association between IL-6 production and UTI severity.^{27, 28} In a model of complicated UTI in which mice underwent urethral obstruction following transurethral UPEC inoculation, *IL-6* knockout (KO) animals experienced higher mortality rates, and survivors demonstrated higher renal UPEC burden and worse renal inflammation than controls.²⁹ The functional significance of IL-6 during uncomplicated cystitis and acute pyelonephritis, however, remains unexplored.

Moreover, the signaling pathways and transcriptional targets activated by IL-6 have not been evaluated during UTI. Following IL-6 binding to its receptor, the Signal Transducer and Activator of Transcription (Stat) 3 undergoes Jak-dependent tyrosine phosphorylation, leading to homodimerization and translocation to the nucleus, where phosphorylated (p)-Stat3 regulates transcription.^{30, 31} Evidence in human immunodeficiency syndromes and mouse models supports a critical role for Stat3 in defending epithelial surfaces against

bacterial pathogens.^{32–35} Stat3 transcriptional targets encode a variety of molecules with roles in antibacterial defense, including antimicrobial peptides (AMPs).^{33, 35}

AMPs promote immunity through bactericidal, bacteriostatic, and immunomodulatory activities.^{36–38} AMP deficiency increases host UTI susceptibility, but little is known regarding signaling pathways that regulate AMP production during UTI. IL-6 and Stat3 have been implicated in transcriptional regulation of multiple AMPs (including *Camp*, *Hamp*, *Lcn2*, *RegIIIγ*, and *RegIIIβ*) by epithelial cells outside of the urinary tract^{33, 35, 39–44} We hypothesized that IL-6 induction by UPEC triggers p-Stat3 dependent AMP expression during UTI, and that genetic deletion of *IL-6* or *Stat3* would impair the host antimicrobial response. We tested this hypothesis in mouse models of experimental UTI initiated by UPEC.

Results

Urinary IL-6 secretion, urothelial Stat3 phosphorylation, and AMP expression during UTI

We investigated the temporal relationship between urinary IL-6 secretion, bladder p-Stat3 levels, and AMP expression in a murine model of UPEC cystitis. Following transurethral UPEC inoculation, urinary IL-6 secretion peaked 2 hours post inoculation (hpi) and returned to near baseline by 16 hpi (Figure 1A). Bladder p-Stat3 levels increased within 2 hpi, peaked at 6 hpi, and returned to baseline by 72 hpi. Total Stat3 levels were unaffected (Figure 1B). P-Stat3 localized to basal and intermediate bladder urothelial cell nuclei (Figure 1C).

Next, we quantified transcript levels of putative IL-6/Stat3 target genes encoding AMPs in bladder RNA extracts. *RegIIIγ*, *RegIIIβ*, *Lcn2*, and *Hamp* mRNA levels were significantly increased within 6 to 16 hpi, coinciding with peak bladder pStat3 levels (Figure 1D). *RegIIIγ* and *RegIIIβ* localized to infected urothelium (Figure 1E).⁴⁵ Thus, experimental UTI initiates a temporal sequence of urinary IL-6 secretion, urothelial Stat3 phosphorylation with nuclear localization, and AMP transcription.

Stat3 phosphorylation and AMP production are IL-6 dependent

We tested the role of IL-6 in Stat3 phosphorylation and AMP expression by performing experimental UTI in *IL-6* knockout (KO) mice. We observed significant reduction in p-Stat3 levels in *IL-6* KO bladders 6 hpi compared to wild type (WT) controls, whereas total Stat3 expression was unchanged (Figure 2A). Similarly, urothelial p-Stat3 immunoreactivity was absent in *IL-6* KO bladders at 6 hpi (Figure 2B). Since IL-6 is required for urothelial Stat3 phosphorylation, we predicted that *IL-6* deficiency leads to reduced expression levels of putative Stat3 transcriptional targets. Accordingly, we investigated expression of Stat3 target genes encoding AMPs in uninfected and infected bladders from *IL-6* KO and WT animals. Uninfected *IL-6* KO and WT bladders expressed comparable AMP mRNA levels (data not shown). At 6hpi, however, *IL-6* KO bladders expressed significantly lower *RegIIIγ*, *RegIIIβ*, and *Hamp* mRNA levels compared to WT (Figure 2C). Another putative IL-6/Stat3 transcriptional target, *Lcn2*, trended lower in *IL-6* KO mice but did not reach statistical significance. We confirmed reduced urinary *RegIIIγ* protein levels in *IL-6* KO mice by

ELISA (Figure 2D). Thus, IL-6 is necessary for urothelial Stat3 phosphorylation and AMP transcription during UTI.

IL-6 drives Stat3 phosphorylation and AMP expression

Next, we tested the direct effect of IL-6 on Stat3 phosphorylation in cultured urothelial cells. We identified brisk induction of p-Stat3 in primary human urothelial cells following IL-6 exposure (Figure 3A), and observed similar responses in transformed human and murine urothelial lines (Figure S1A). In contrast, we observed little or no IL-6 and p-Stat3 induction following UPEC incubation in these same cell lines (Figure S1B). Next, we investigated whether IL-6 administration is sufficient to drive Stat3 phosphorylation and AMP mRNA expression *in vivo* in the absence of UTI. Intraperitoneal injection of recombinant, murine IL-6 led to p-Stat3 in bladders and kidneys (Figure 3B). In addition, there was significant induction of *Lcn2*, *RegIII β* , and *RegIII γ* AMP mRNA levels, though not to the magnitude observed during experimental UTI (Figure 3C). Thus, systemic IL-6 administration in the absence of UTI is sufficient to induce bladder p-Stat3 and AMP mRNA expression.

Impaired bladder AMP mRNA expression in Stat3 knockout mice

To directly test the role of Stat3 in AMP mRNA expression, we performed experimental UTI in *Stat3* conditional knockout mice. To delete *Stat3* throughout the urothelium, we made use of *UBC-CreERT2*; *Stat3^{fl/fl}* mice (*Stat3*^{-/-} mice), in which expression of a tamoxifen-inducible Cre recombinase is driven by the constitutive *UBC* promoter.⁴⁶ Tamoxifen-treated Cre(-); *Stat3^{fl/fl}* littermates were used as controls (*Stat3^{fl/fl}* mice). We confirmed Cre-mediated recombination following tamoxifen administration by RT-PCR (Figure 4A) based on detection of a truncated *Stat3* transcript.⁴⁷ Western blotting demonstrated markedly reduced p-Stat3 and total Stat3 protein levels in UPEC infected *Stat3*^{-/-} bladders (Figure 4B). Loss of Stat3 led to significant reduction in *Hamp*, *RegIII γ* , and *RegIII β* mRNA levels in *Stat3*^{-/-} bladders 6 hpi (Figure 4C) and undetectable RegIII γ protein in *Stat3*^{-/-} urine 6hpi (Figure 4D). Thus, Stat3 is required for AMP mRNA expression during experimental UTI.

IL-6 deficiency leads to increased bladder epithelial cell invasion and bacteriuria following experimental cystitis

To determine the consequence of *IL-6* deficiency on UPEC clearance during acute cystitis, we measured bacterial burden in *IL-6* KO versus WT tissues following experimental UTI. We observed significant increases in urine bacterial burden in *IL-6* KO mice at 24, 48, and 72 hpi (Figure 5A). We observed a transient increase in UPEC burden recovered from *IL-6* KO bladders 6 hpi that resolved by 24 hpi (Figure 5B). In contrast, *IL-6* deficiency did not lead to significant alterations in renal bacterial burden, nor did we observe increased mortality in *IL-6* KO as reported previously in obstructed infected mice.²⁹

To clarify the early difference in bladder colonization between *IL-6* KO and WT controls, we enumerated intracellular bacterial communities (IBC). IBC number increased significantly in *IL-6* KO at 6 and 16 hpi, normalizing by 24 hpi (Figure 5C). The increase in IBC number 6 hpi was reversed by intravesical delivery of recombinant murine IL-6 (Figure 5D). We also observed increased IBC numbers in *Stat3*^{-/-} mice 6 hpi (Figure 5E), consistent with a role for IL-6/Stat3 signaling in regulating intracellular UPEC *in vivo*.

IL-6 neutralization leads to increased renal bacterial burden and inflammation

We reasoned that the genetic background of *IL-6* KO mice (C57BL/6J) limits the proclivity of these animals to acute pyelonephritis. Therefore, we evaluated the impact of IL-6 neutralization on pyelonephritis susceptibility in C3H/HeOuJ mice, which develop pyelonephritis at a high frequency after transurethral inoculation of UPEC despite an intact *Tlr4* locus.^{27, 48} Systemic delivery of an IL-6 neutralizing antibody (α -IL-6) led to reduced IL-6 levels and decreased bladder and kidney p-Stat3 in response to UPEC, compared to animals treated with isotype control antibody (α -HRPN; Figure 6A and 6B). IL-6 neutralization led to increased renal bacterial burden 7 days post infection (dpi; Figure 6C), accompanied by renal abscess formation, neutrophil casts, and interstitial hemorrhage (Figure 6D).

Stat3 phosphorylation and AMP production during UTI requires intact Tlr4 signaling

Given the requirement for Tlr4 signaling in IL-6 secretion during cystitis,^{13, 16, 18} we hypothesized that Stat3 phosphorylation and AMP mRNA expression display Tlr4 dependence. In order to evaluate this, we compared IL-6 production, p-Stat3, and AMP mRNA levels in infected C3H/HeOuJ (Lps-sensitive) and C3H/HeJ (Lps-resistant) mice during experimental UTI. UPEC infection did not elicit urinary or systemic IL-6 secretion in C3H/HeJ animals (Figure 7A). In addition, p-Stat3 levels were attenuated in infected C3H/HeJ bladders and kidneys compared to C3H/HeOuJ controls (Figure 7B), whereas total Stat3 protein levels were unchanged. While AMP mRNA levels did not differ at baseline between the strains in the absence of UPEC infection (data not shown), putative Stat3 transcriptional target AMPs *RegIII γ* , *RegIII β* , *Lcn2*, *Hamp*, and *Camp* were reduced in C3H/HeJ bladders 24 hpi (Figure 7C). Thus, Tlr4 signaling regulates Stat3 phosphorylation and mRNA expression of AMPs during UTI.

Discussion

While IL-6 secretion has been associated with clinical and experimental UTI for decades,^{16, 27, 28} the functional significance and mechanistic action of IL-6 during acute cystitis have remained elusive. Our data support a model in which UPEC signals through Tlr4 to elicit IL-6 production, which triggers urothelial Stat3 phosphorylation and AMP expression. Of note, we identify a novel role for IL-6/Stat3 signaling in urothelial susceptibility to IBC formation, demonstrating that deletion of *IL-6* or *Stat3* leads to increased number of IBCs. In addition, neutralization of IL-6 leads to increased renal bacterial burden and severe inflammation. These findings have implications for our understanding of UTI pathogenesis and the roles of IL-6/Stat3 signaling in the host innate immune response to UTI.

Tlr4-dependent IL-6 secretion links UPEC infection to Stat3 phosphorylation and AMP expression

In this study, we found that Stat3 phosphorylation and AMP mRNA induction during cystitis depend on intact Tlr4 signaling. We were unable to elicit robust Stat3 phosphorylation in urothelial cells treated with live UPEC or Lps alone (Figure S1A and data not shown), however, arguing that Stat3 phosphorylation does not arise as a direct consequence of Tlr4 signaling. Rather, we demonstrate that IL-6 serves a critical role in triggering urothelial

Stat3 phosphorylation and AMP mRNA expression during cystitis. Previous work has established that secretion of IL-6 by bladder urothelial cells is Tlr4-dependent, since inhibition of Lps or Tlr4 signaling results in impaired IL-6 production following *E. Coli* treatment.¹⁸ While UPEC strains are capable of suppressing IL-6 production by urothelial cell lines *in vitro*,^{19, 20} our *in vivo* data are consistent with a model in which bacterial endotoxin signals through Tlr4 to activate IL-6 secretion, leading to Stat3 phosphorylation and AMP transcription. In this manner, IL-6 secretion may serve to amplify the host response to UPEC by triggering antimicrobial defense mechanisms in surrounding urothelial cells.

IL-6/Stat3 regulates IBC number with implications for UPEC virulence

The importance of IL-6/Stat3 signaling is highlighted by our finding of increased IBC numbers in *IL-6* KO and *Stat3* KO bladders, along with the demonstration that intravesical IL-6 administration reduces IBC number in *IL-6* KO bladders. IL-6 elicitation by UPEC treated urothelial cells has been previously shown to vary inversely with virulence, suggesting that UPEC fitness in the urinary tract may be achieved in part by blunting the initial burst of IL-6 production.²¹ Our findings support this concept, as a reduction in IL-6 secretion should limit Stat3 phosphorylation by urothelial cells – favoring UPEC invasion and increased IBC formation, as observed in *IL-6* KO and *Stat3* KO mice. These findings parallel the observations of Schilling and coworkers, who demonstrated that Tlr4-hyporesponsive C3H/HeJ mice had large foci of intracellular bacteria in the absence of a normal inflammatory response.¹⁸ Together, these findings are consistent with a model in which Tlr4/IL-6/Stat3-dependent signaling limits IBC formation in the urothelium. To our knowledge, this pathway encompasses the only known host genes implicated in controlling IBC number.

Impact of the IBC bottleneck on ascending infection and chronic UTI

IBC formation represents a significant population bottleneck in the UPEC life cycle during cystitis, with only 0.01-0.001% of the initial UPEC inoculum contributing to IBC formation.⁴⁹ Thus, mechanisms that facilitate increased IBC formation are associated with higher UPEC virulence, leading to UTI chronicity and upper tract infection.⁴⁹ Indeed, Schwartz and coworkers demonstrated that mice that developed persistent bacteriuria and chronic cystitis had increased bacterial diversity reflecting increased IBC formation during acute infection compared to those mice able to clear infection two weeks after inoculation.⁴⁹ We hypothesize that increased IBC numbers - arising from host deficiency in *IL-6* or *Stat3* or via UPEC suppression of IL-6 production - effectively widen the population bottleneck, increasing UPEC diversity and heightening host susceptibility to chronic UTI and ascending infection.⁵⁰

Functional significance of AMP production during UTI

We identify AMP production as one antibacterial mechanism impacted by *IL-6* and *Stat3* deficiency. In particular, this study identified three AMPs as transcriptional targets of IL-6/Stat3 signaling during UTI: Hepcidin, RegIII β , and RegIII γ . Hepcidin, encoded by the *Hamp* gene, exhibits bacteriostatic activity toward *E. coli*, and its deficiency leads to increased renal UPEC burden, but the consequence on IBC formation was not evaluated in

this study.⁵¹ RegIII β exhibits bactericidal activity against Gram-negative bacteria and is implicated in clearance of salmonellosis *in vivo*,⁵² but its function has not yet been evaluated during UTI. In contrast, RegIII γ antimicrobial activity is restricted to Gram-positive organisms, and its deficiency did not impact UPEC burden, though IBC number was not evaluated. Further studies are required to determine if higher IBC numbers in infected *IL-6* and *Stat3* KO mice arise as a consequence of reduced AMP production. While it is conceivable that a collective deficiency in AMP production favors increased IBC formation, it is equally plausible that other downstream targets of IL-6/Stat3 signaling account for this phenotype.

Conclusions

We have implicated IL-6/Stat3 signaling in urothelial AMP production and inhibition of IBC formation during UTI. Based on our observations, we speculate that genetic or acquired deficiencies in IL-6/Stat3 signaling may lead to increased susceptibility to recurrent UTI and pyelonephritis due to alterations in IBC formation and AMP production. In this regard, it is noteworthy that genitourinary infections are a recognized side effect of tocilizumab, a humanized monoclonal antibody directed toward IL-6.⁵³ Conversely, strategies that trigger IL-6/Stat3 signaling may promote AMP production, limit IBC formation, and hasten UTI resolution.

Methods

Mice and Genotyping

Experiments were conducted with the approval of our Institutional Laboratory Animal Care and Use Committee (Welfare Assurance Number A3544-01), Protocol AR13-00057. All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Except for the C3H/HeOuJ and C3H/HeJ strains, all animals were on a pure C57BL/6J background. Animals were PCR genotyped according to the company website. To confirm Cre/LoxP recombination in *Stat3* mice, we performed RT-PCR to detect the truncated *Stat3* mRNA in which exons 18–20 are deleted, using primers 5'-TGGGCATCAATCCTGTGGTAT-3' and 5'-GCTGCTGCTTGGTGTATGGCTCTA-3', which generate a 514 bp product (flox) or 226 bp product ().

Experimental UTI

One week prior to experimental UTI, *Stat3* and *Stat3*^{flox} mice received three 100 μ l intraperitoneal injections of tamoxifen (Sigma, 20 mg/kg in corn oil); each injection was spaced 48 hours apart. Experimental UTI was induced in female mice age 6–8 weeks with UPEC strain CFT073, isolated from the blood and urine of a patient with pyelonephritis.⁵⁴ CFT073 was inoculated from a glycerol stock and grown statically in LB medium for 16 hours at 37°C. The inoculum was 10⁸ colony forming units (CFU) in 50 μ l phosphate buffered saline (PBS). After infection, tissue was isolated and bacterial burden assessed.^{55, 56} We followed a published protocol for IBC enumeration.⁴⁹

IL-6 Administration and Neutralization

For systemic administration in the absence of UTI, 100 ng murine IL-6 (Peprotech, Rocky Hill, NJ) or PBS/0.1% bovine serum albumin carrier was injected intraperitoneally in 100 μ l total volume. For local administration during UTI, 100 ng IL-6 or carrier was injected intravesically in 50 μ l one hour before UPEC inoculation and repeated 2 hours after inoculation. For neutralization, female C3H/HeOuJ mice received an intraperitoneal injection of 1 mg anti-mouse IL-6 (BioXCell, clone MP5-20F3, in 200 μ l) or rat IgG1 isotype control (BioXCell, clone HRPN) 24 hours prior to UPEC infection. The injection was repeated 1, 3, and 6 dpi.

ELISA

Commercial ELISAs detected IL-6 (R&D Systems, Minneapolis, MN) and RegIII γ (Cloud-Clone Corporation, Katy, TX). Urine IL-6 and RegIII γ levels were normalized to urine creatinine (Cr) in a colorimetric assay (Oxford Biomedical Research, Rochester Hills, MI).

RNA Extraction and QRT-PCR

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). Quantitative (q)RT-PCR was performed using gene-specific primer pairs (Table 1) and SybrGreen.^{45, 57} Relative expression changes were calculated by the $2^{-\Delta\Delta CT}$ method, normalizing to a pool of uninfected, wild-type, bladder or kidney cDNA.⁵⁸

Western Blotting and Immunostaining

Protein extraction, western blotting, and immunostaining were performed as described. Primary antibodies were purchased from Cell Signaling (Beverly, MA) except for rabbit α -RegIII γ (Dr. Lora Hooper, University of Texas at Southwestern) and sheep α -RegIII β (R&D systems). Primary antibodies were detected with AlexaFluor 488- or AlexaFluor 594-conjugated secondary antibodies raised in donkey (Jackson ImmunoResearch, West Grove, PA). Negative controls sections were incubated with irrelevant species-specific antibody or secondary antibody alone.

Primary Urothelial Cells and Cell Lines

Human primary bladder epithelial cells (ATCC, Manassas, VA) were grown in serum-free, prostate epithelial cell basal medium with supplements. Human 5637 cells were purchased from ATCC and cultured in RPMI with L-glutamine and 10% fetal bovine serum. MBT-2 and MB-49 cells (Dr. Timothy Ratliff, Purdue University) were cultured in DMEM with 10% fetal bovine serum, 4.5 g/L glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate. Cells were serum-starved 24 hours before experiments. Recombinant IL-6 (R&D Systems) was added to a final concentration of 25 ng/ml. For UPEC stimulation, overnight static cultures of CFT073 were diluted based on OD600 to multiplicity of infection of 20.

Statistical Analysis

Mann-Whitney U test compared bacterial burden, IL-6 and RegIII γ protein levels, and relative mRNA expression levels (Graph Pad, La Jolla, CA). For comparisons involving

more than two groups, the Kruskal-Wallis test was used. *p* values of < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental UTI triggers IL-6 secretion, urothelial p-Stat3, and AMP mRNA expression

A) Urine IL-6/Cr levels peak 2 hpi following transurethral UPEC inoculation. Urine was pooled from 6 mice/experimental group at each time point. Bars indicate mean $\pm$ standard deviation of triplicate IL-6/Cr values. #: $p = 0.0073$, Kruskal-Wallis test. **B)** Western blotting demonstrates a transient increase in p-Stat3 levels in UPEC infected bladders. **C)** Immunofluorescence microscopy identifies p-Stat3 reactive nuclei (pink staining) localized mainly within the basal and intermediate urothelial layers. Green staining demonstrates E-cadherin, and DAPI-positive nuclei are indicated in blue. The white dotted line indicates the basement membrane. Scale bars represent 16.25 microns, 60x original magnification. **D)** QRT-PCR identifies bladder AMP mRNA induction during experimental UTI. * $p < 0.05$, compared to uninfected, Mann-Whitney U test, $n=4$ bladders/time point. **E)** RegIII β and RegIII γ proteins (green) localize to the urothelium during urinary tract infection. Scale bars represent 16.25 microns (RegIII β) and 25 microns (RegIII γ), 60x and 40x original magnification, respectively.

Figure 2. IL-6 is necessary for urothelial Stat3 phosphorylation and AMP mRNA production following UTI

A) Western blot detects attenuated p-Stat3 induction 6hpi in *IL-6* KO bladders compared to C57BL/6J controls. Total Stat3 is unchanged in both strains. **B)** Immunofluorescence demonstrates the absence of p-Stat3 reactivity in *IL-6* KO urothelium 6 hpi, compared to C57BL/6J controls. Positive p-Stat3 reactivity is marked by pink nuclear staining and highlighted by arrows. Green: E-cadherin; Blue: nuclei; scale bars represent 25 microns, 40x original magnification. **C)** Impaired expression of *RegIIIβ*, *RegIIIγ*, and *Hamp* transcripts in *IL-6* KO bladders 6 hpi compared to WT C57BL/6J mice; * $p = 0.0379$ and # $p = 0.0002$ Mann-Whitney U test, $n = 8$ mice/group. **D)** Reduced RegIIIγ/Cr levels in *IL-6* KO versus WT C57BL/6J urine 24 hpi. # $p = 0.0286$, Mann-Whitney U test, $n = 6$ mice/group.

Figure 3. IL-6 drives urothelial Stat3 phosphorylation and AMP production in the absence of UTI

A) Primary human urothelial cells exhibit p-Stat3 induction in response to recombinant human IL-6 treatment. **B)** Intraperitoneal injection of 100 ng recombinant murine IL-6 induces p-Stat3 in WT bladders and kidneys within 1 hour as compared to animals injected with PBS carrier alone. **C)** IL-6 treatment induces *Lcn2*, *RegIIIβ*, and *RegIIIγ* mRNA in the absence of UTI. Animals underwent intraperitoneal injection of IL-6 or carrier, and were euthanized 4 hours later. * $p = 0.0023$ and # $p = 0.007$, Mann-Whitney U test. Data shown represent 4 mice/condition from one of two independent experiments.

Figure 4. AMP expression is significantly reduced in *Stat3* mice

A) RT-PCR demonstrates unique detection of a truncated *Stat3* mRNA in *Stat3* bladders after tamoxifen injection. **B)** Reduced baseline total Stat3 protein levels as well as total Stat3 protein and pStat3 at 6 hpi in *Stat3* and *Stat3^{flox}* bladders; **C)** Significantly decreased *Hamp*, *RegIIIβ*, and *RegIIIγ* mRNA levels in *Stat3* bladders 6 hpi versus *Stat3^{flox}* controls (* $p = 0.0022$, Mann-Whitney U test). $n = 6$ mice/condition from two independent experiments. **D)** RegIIIγ protein secretion is undetectable in *Stat3* urine 6 hpi, compared to *Stat3^{flox}* controls; n.d. = not detectable; # $p = 0.03$, student's t test, $n = 6$ mice/group from two independent experiments.

Figure 5. IL-6 deficiency leads to increased bacteriuria and IBC formation

A) Higher magnitude of UPEC recovered from *IL-6* KO urine compared to WT C57BL/6J controls. $*p=0.0021$; $**p=0.0440$; $\#p=0.0045$, Mann-Whitney U test. **B)** Transient increase in bladder UPEC burden 6 hpi in *IL-6* KO ($\#p=0.038$, Mann-Whitney U test), that was not subsequently sustained. **C)** Increased IBC in *IL-6* KO bladders 6 and 16 hpi compared to WT C57BL/6J controls, that normalizes by 24 hpi. $*p=0.0043$; $**p=0.0286$, Mann-Whitney U test. **D)** This increased IBC phenotype in *IL-6* KO bladders is diminished in the setting of recombinant IL-6 protein given intravesically 1 hour before infection and again 2 hpi prior to mice being sacrificed at 6hpi. $*p=0.0317$, Mann-Whitney U test. **E)** There is a similar increase in IBC in *Stat3*^Δ bladders compared to control *Stat3*^{flox} bladders. $*p=0.0216$, Mann-Whitney U test.

Figure 6. IL-6 neutralization in C3H/HeOuJ mice leads to severe pyelonephritis

A) Reduction in serum IL-6 levels induced by UPEC in C3H/HeOuJ mice pretreated with α -IL-6 neutralizing antibody 24 hours before infection versus isotype control antibody (α -HRPN). * $p < 0.05$, Kruskal-Wallis test. $n = 8$ mice/group. **B)** Reduced bladder and kidney p-Stat3 induction by UPEC in C3H/HeOuJ mice treated with α -IL-6 neutralizing antibody versus isotype control (α -HRPN), at specified times after inoculation. **C)** Increased renal bacterial burden in mice treated with α -IL-6 neutralizing antibody versus α -HRPN 7 days post UPEC infection. # $p = 0.036$, Mann-Whitney U test. **D)** H&E staining of a representative kidney following α -IL-6 neutralizing antibody treatment (left) demonstrating extensive cortical inflammation 7 dpi. The middle micrograph shows higher power magnification of the area depicted in the inset with areas of neutrophilic infiltrate, neutrophil casts in tubules (white arrows), hemorrhage (white asterisk), and abscess (a) formation. An unaffected glomerulus (g) is visible. An example of a representative kidney following α -HRPN treatment is shown for comparison (right) which demonstrates normal renal architecture. Scale bars represent 200 microns, 4x and 20x original magnification.

Figure 7. IL-6 production, Stat3 phosphorylation, and AMP production are Tlr4-dependent
A) UPEC inoculation in LPS-responsive C3H/HeOuJ mice results in urine IL-6 secretion and accumulation of serum IL-6 24 hpi compared to uninfected (Un) controls. Urine and serum IL-6 was not detected (n.d.) in LPS resistant C3H/HeJ mice at the same time point (* $p = 0.0046$; ** $p = 0.0015$, # $p = 0.034$, ## $p = 0.001$, one-way ANOVA, $n = 6$ mice/group [urine] or 4 mice/group [serum]). **B)** Western blotting indicates UPEC induced p-Stat3 expression in C3H/HeOuJ bladders and kidneys to a greater extent than that of C3H/HeJ animals. **C)** Reduced bladder mRNA levels of AMPs in C3H/HeJ bladders compared to C3H/HeOuJ mice 24 hpi (* $p < 0.01$; $n = 5$ mice/group).

Table 1

QRT-PCR Primers

Gene	Forward	Reverse
<i>Camp</i>	5'-TTCAAGGAACAGGGGGTGG-3'	5'-AGGCTCGTTACAGCTGATGTC-3'
<i>Defb14</i>	5'-CATTCCTACCAAAAACCTC-3'	5'-CTTCTACTTCTTTCGGC-3'
<i>Gapdh</i>	5'-CTGGAGAAACCTGCCAAGTA-3'	5'-TGTTGCTGTAGCCGTATTCA-3'
<i>Hamp</i>	5'-GAGCAGCACACCTATCTCC-3'	5'-TTGGTATCGCAATGTCTGCC-3'
<i>Lcn2</i>	5'-ATATGCACAGGTATCCTCAG-3'	5'-GAAACGTTCTTCAGTTCAG-3'
<i>RegIII β</i>	5'-GTCAAGAGCTTCTGGATTTC-3'	5'-GCTTTTCTAGGTGAGTCTTC-3'
<i>RegIII γ</i>	5'-GACAAGATGCTTCCCGTAT-3'	5'-GGCATCTTCTTGCCAACCTT-3'