

Murine Ribonuclease 6 Limits Bacterial Dissemination during Experimental Urinary Tract Infection

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Keywords

Antimicrobial peptide · Antimicrobial protein · Bacterial infection · Monocyte · Macrophage · Host defense · Urinary tract infection · Cystitis · Pyelonephritis

Abstract

Introduction: The ribonuclease (RNase) A superfamily encodes cationic antimicrobial proteins with potent microbicidal activity toward uropathogenic bacteria. Ribonuclease 6 (*RNase6*) is an evolutionarily conserved, leukocyte-derived antimicrobial peptide with potent microbicidal activity toward uropathogenic *Escherichia coli* (UPEC), the most common cause of bacterial urinary tract infections (UTIs). In this study, we generated *RNase6*-deficient mice to investigate the hypothesis that endogenous RNase 6 limits host susceptibility to UTI. **Methods:** We generated a *RNase6*^{EGFP} knock-in allele to identify cellular sources of *RNase6* and determine the consequences of homozygous *RNase6* deletion on antimicrobial activity and UTI susceptibility. **Results:** We

identified monocytes and macrophages as the primary cellular sources of *RNase6* in bladders and kidneys of *RNase6*^{EGFP/+} mice. *RNase6* deficiency (i.e., *RNase6*^{EGFP/EGFP}) resulted in increased upper urinary tract UPEC burden during experimental UTI, compared to *RNase6*^{+/+} controls. UPEC displayed increased intracellular survival in *RNase6*-deficient macrophages. **Conclusion:** Our findings establish that *RNase6* prevents pyelonephritis by promoting intracellular UPEC killing in monocytes and macrophages and reinforce the overarching contributions of endogenous antimicrobial RNase A proteins to host UTI defense.

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Published by S. Karger AG, Basel

Introduction

Bacterial urinary tract infections (UTIs) afflict 400 million people annually, including 50–60% of women, of whom 25–30% suffer UTI recurrence [1–3]. Uropathogenic

Escherichia coli (UPEC) is the leading cause of UTI, accounting for 80–90% of cases [3, 4]. While most UTIs are confined to the bladder (cystitis), the risk of ascending UTI, pyelonephritis (PN), and urosepsis is increased in a host of clinical settings including infants and young children, immunocompromised patients, individuals with neurogenic bladder, chronic indwelling catheter use, and the elderly [5–8]. Not only can PN result in acute kidney injury, but 15% of young children with PN will develop renal scars [9], which can lead to hypertension, proteinuria, and chronic kidney disease. There is an urgent need for alternative and more effective strategies to treat UTI and prevent its sequelae.

A better understanding of the host immune response should uncover strategies to prevent UTI. To destroy invading uropathogens, the innate immune system relies on a potent combination of phagocyte recruitment, complement activation, and production of antimicrobial factors, including antimicrobial peptides and proteins (AMPs) [10, 11]. AMPs are evolutionarily conserved, cationic peptides with multiple properties that confer antimicrobial activity – including disruption of microbial membranes, sequestration of key micronutrients, disabling virulence factors, blocking cell division, and impairing translational machinery [12]. A growing number of AMPs have been implicated in host defense against UTI based on their potent antimicrobial activity toward uropathogens and increased UTI susceptibility in AMP-deficient mice [13–18]. Multiple innate immune cell types produce AMPs, including urothelial cells of the lower urinary tract, principal and intercalated cells within the kidney's collecting ducts, neutrophils, monocytes, and macrophages [10–12].

The ribonuclease (RNase) A superfamily includes multiple AMPs with broad-spectrum antimicrobial activity toward Gram-negative and Gram-positive bacteria as well as fungi [19, 20]. Work from our group has established key roles for epithelial RNase 4 and RNase 7 in host defense against UTI. Antibody neutralization of RNase 4 and RNase 7 results in increased growth of UPEC in urine [15, 21]. Expression of human RNase 7 in transgenic mice or uroepithelial cells leads to increased eradication of UPEC, while *RNASE7* depletion is associated with increased UPEC attachment and invasion of uroepithelial cells [22]. In contrast, far less is known regarding the contributions of leukocyte-derived RNases to host defense during UTI.

RNase 6 is an evolutionarily conserved AMP expressed by leukocytes in patients with UTI and during murine experimental UTI [23]. Human and mouse RNase 6 kill UPEC at low micromolar concentrations

without exerting cytotoxicity toward host cells [23, 24]. Recently, we determined that human *RNASE6* transgenic mice are protected from experimental UTI, consistent with the concept that future interventions to augment the level and/or activity of RNase 6 may reduce the incidence and severity of UTI [25, 26]. In this study, we generated mice with a *Rnase6*^{EGFP} knock-in allele to study the cellular sources of *Rnase6* along with the consequences of its deletion on UPEC killing and susceptibility to experimental UTI.

Methods

Study Approval

Animal experiments were approved by and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee at Abigail Wexner Research Institute at Nationwide Children's Hospital.

Generation of *Rnase6*^{EGFP} Knock-In Mice

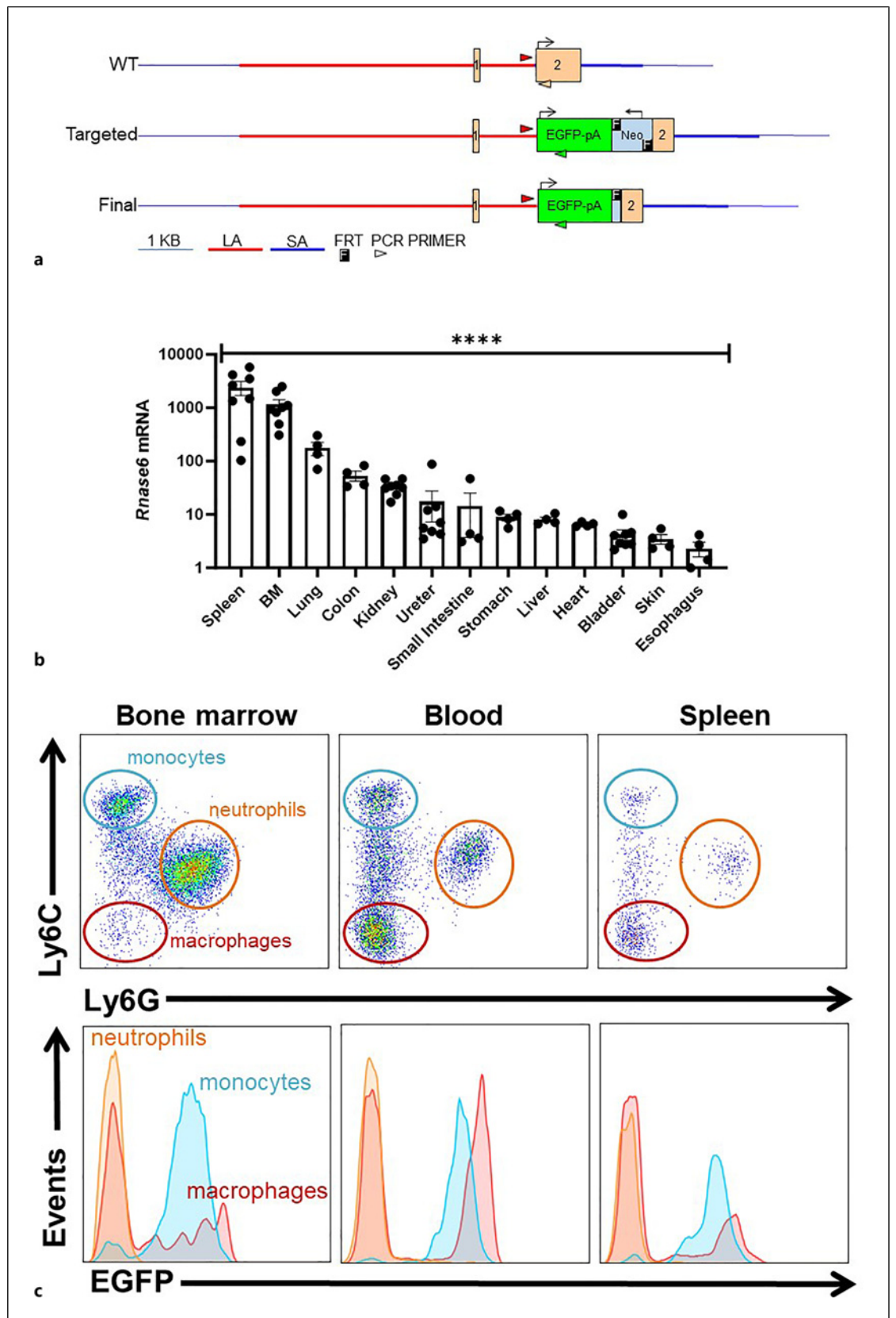
Rnase6^{EGFP/+} knock-in mice were generated by InGenious Targeting Laboratory (Ronkonkoma, NY, USA). A targeting vector containing a frt-flanked neomycin resistance (Neo) cassette was used to introduce the EGFP followed by a polyadenylation signal into exon 2 of the *Rnase6* gene, beginning at the *Rnase6* start codon ("Targeted," Fig. 1a). The EGFP replaced the first 61 amino acids of the RNase 6 protein and was followed by a polyadenylation signal leading to a truncated transcript that omits the remainder of exon 2. A frameshift mutation provided an additional safeguard against production of a C-terminal RNase 6 fragment. The construct was electroporated into C57BL/6-derived iTL BF1 embryonic stem cells (C57BL/6 FLP) which express FLP recombinase to remove the Neo cassette ("Final," Fig. 1a). Correctly targeted embryonic stem cells were injected into BALB/c blastocysts. The resulting chimeric animals were bred to C57BL/6 wildtype (WT) mice. The *Rnase6*^{EGFP} and WT *Rnase6* alleles were distinguished by PCR using the following primers: 5'-ACAATCAGGCCAGCC TACTCG-3' (common forward), 5'-ACCGCGCATGGCTGT GTT-3' (*Rnase6* reverse), and 5'-GCTCCTCGCCCTTGCTCAC-3' (*Rnase6*^{EGFP} reverse), yielding a 370 bp (*Rnase6*^{EGFP}) or 200 bp (*Rnase6* WT) product.

Characterization of *Rnase6*^{EGFP/EGFP} and *Rnase6*^{+/+} Mice

Serum chemistries, complete blood counts, and baseline organ histology in healthy, age-matched *Rnase6*^{EGFP/EGFP} and *Rnase6*^{+/+} female mice ($n = 4\text{--}6/\text{group}$) were evaluated through the Ohio State University Comparative Pathology and Mouse Phenotyping Shared Resource.

QRT-PCR

Isolation of total RNA, reverse transcription, and quantitative PCR detection were performed as described [25]. Results were analyzed by the $2^{-\Delta\Delta CT}$ method, normalizing to *Gapdh*. Primers used were *Rnase6* forward 5'-TGGCCCTGTTACCATAGGAG



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CC-3' and reverse 5'-GCGCATGGCTGTGTTGCATGG-3'; and *Gapdh* forward 5'-CTGGAGAAACCTGCCAAGTA-3' and reverse 5'-TGTTGCTGTAGCCGTATTCA-3'.

Immunofluorescence Microscopy

Formalin-fixed, paraffin-embedded kidneys were sectioned at 4 μ m. Following citrate-based antigen retrieval, immunolocalization was performed using the following primary antibodies: chicken α -GFP (1:300; Abcam, Waltham, MA, USA); rabbit α -keratin 7 (1:500; Novus, Centennial, CO, USA); and goat α -red fluorescent protein (RFP) (1:500; Rockland Immunochemicals, Limerick, PA, USA). Cy3- and Alexa Fluor 488-conjugated secondary antibodies raised in donkey were used (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Slides were treated with 0.1% Sudan Black B (Thermo Fisher, Wilmington, DE, USA) to reduce background autofluorescence and incubated in Hoechst (1:2,000; Thermo Fisher). Next, slides were cover slipped using ProLong Gold Antifade Reagent (Invitrogen, Waltham, MA) and imaged at 40 $\times$ using a Nikon Eclipse Ti2 microscope. *Rnase6*^{EGFP/+} tissues were processed in parallel to confirm that α -EGFP reactivity was specific to *Rnase6*^{EGFP/+} tissues. Additional negative control sections were incubated with irrelevant species-specific Ab or secondary Ab alone.

Flow Cytometry

Bladders and kidneys were minced and digested in DMEM/F-12 medium with 1 mg/mL collagenase type 4, 20 mg/mL DNase I, 50 mM HEPES, and 0.5% BSA/fraction V (Invitrogen). After enzymatic dissociation, samples were further disaggregated by passing them through a cell strainer using a syringe plunger. Mononuclear and polymorphonuclear cells from the kidney were enriched using a double gradient formed by layering an equal volume of Histopaque-1077 over Histopaque-1119 (Sigma, St. Louis, MO, USA). Single-cell suspensions were incubated in 1 mg/mL of anti-mouse Fc receptor (anti-mouse anti-CD16/32, clone 93) in 100 μ L phosphate-buffered saline (PBS) containing 0.5% BSA plus 0.02% sodium azide (FACS buffer) for 15 min on ice to block nonspecific Ab binding. To exclude dead cells, samples were then stained with blue-fluorescent reactive dye (Life Technologies) for 20 min at room temperature. After washing, 1–3 million cells were stained for cell-surface receptors in FACS buffer, for 30 min at 4°C, with various fluorescent mAb combinations (see the list below). Flow cytometry studies used Abs at 1:100 dilutions. Stained cells were further collected on an LSR II cytofluorometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Absolute cell numbers were calculated using CountBright Absolute Counting Beads (Thermo Fisher). The following fluorochrome-conjugated Abs used PerCP/Cy5.5 anti-mouse Ly-6G Ab (BioLegend, clone 1A8), Brilliant Violet 650 anti-mouse I-A/I-E Ab (BioLegend, clone M5/114.15.2), Brilliant Violet 785 anti-mouse CD45 Ab (BioLegend, clone 30-F11), Alexa Fluor 700 anti-mouse/human CD11b Ab

(BioLegend, clone M1/70), PerCP/Cy5.5 anti-mouse Ly6G Ab (BioLegend, clone 1A8), PE anti-mouse Cx3cr1 Ab (BioLegend, clone SA011F11), PE anti-mouse CD45 Ab (BioLegend, clone 30-F11), PE/Cy7 anti-mouse Ly6C Ab (BioLegend, clone HK1.4), and eFluorTM 450 anti-mouse Ly-6C Ab (Invitrogen, clone HK1.4).

Experimental UTI

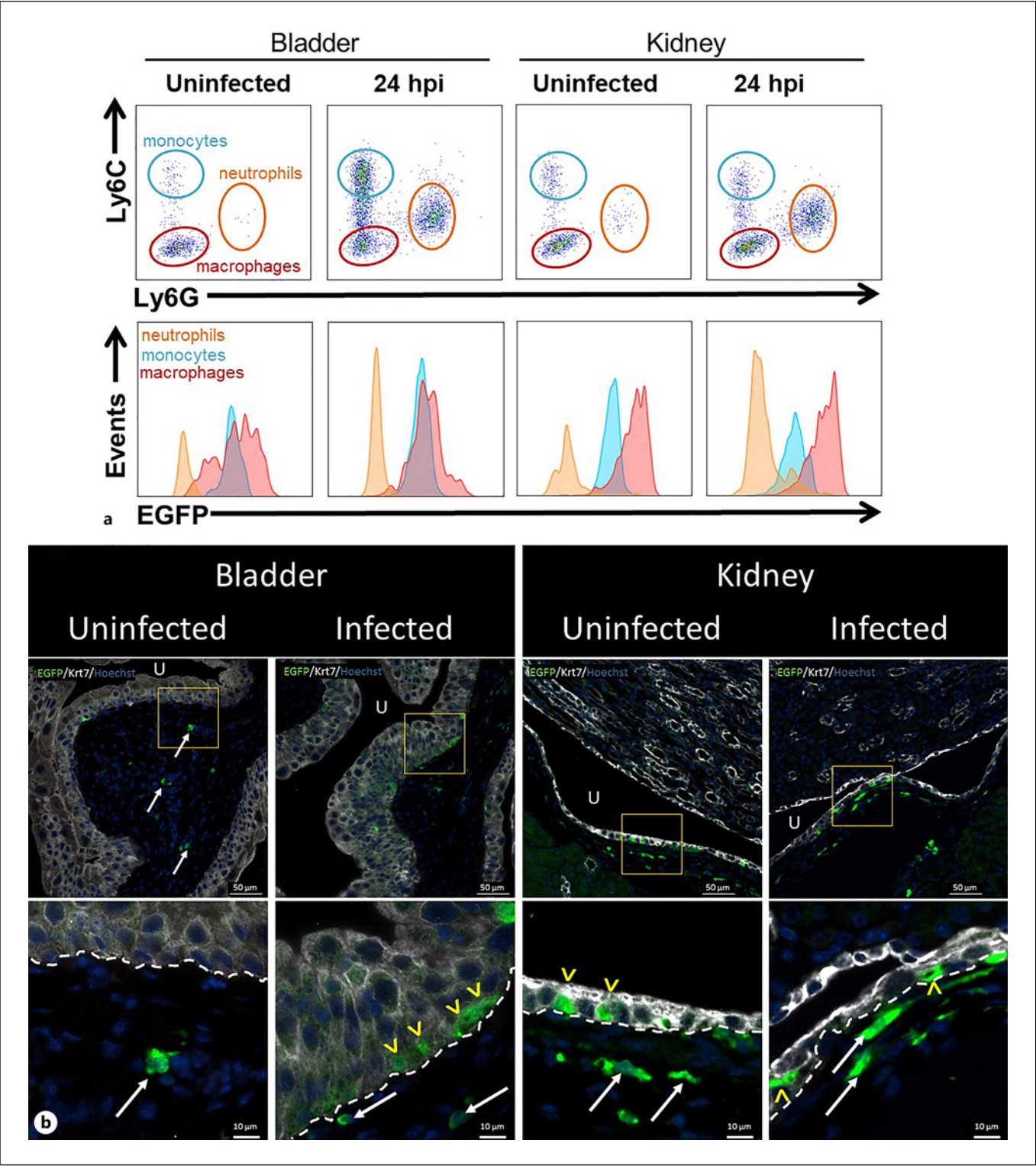
Experimental UTI was induced in anesthetized, 6- to 8-week-old female mice on a C57BL/6J background by transurethral inoculation with CFT073, a clinical UPEC isolate from the blood of a patient with PN [27]. CFT073 was inoculated from a glycerol stock and grown statically in LB medium for 16 h at 37°C. The inoculum was 10⁸ colony-forming units (CFUs) in 50 μ L PBS, based on our prior work and protocols for murine experimental UTI [28–31]. Morbidity was assessed using a standard body condition score and observed behavior [32], in accordance with national and local guidelines for animal wellness. There was no significant morbidity, however, as is generally the case in C57BL/6J mice [33], and no animals met predefined humane endpoint criteria requiring euthanasia. After 3, 6, 12, 24, 48, and 72 h following infection, mice were re-anesthetized and euthanized by exsanguination, urinary tract organs were isolated, and bacterial burden assessed (8–24 mice/genotype/timepoint, details in figure legend) [28]. Alternatively, bladders were fixed in 10% formalin, embedded in paraffin, and H&E stained, and mucosal injury and inflammation were scored by a veterinary pathologist in blinded fashion (5 mice/genotype/condition) [29, 33, 34]. Intracellular bacterial communities were visualized in bladder whole mounts based on β -galactosidase activity (6–7 mice/genotype/condition) and enumerated as described [31, 35]. Briefly, bladders were bisected and played on silica plates and then fixed with 4% paraformaldehyde (Santa Cruz). The fixed bladders were washed with 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40 in sterile PBS, pH 7.4. Bladders were then incubated with 0.4 mL of staining solution (25 mg/mL X-Gal, 1 mM potassium ferrocyanide, and 1 mM potassium ferricyanide) for 16 h at 30°C and visualized under a dissecting microscope.

UPEC Killing by Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages (BMDMs) were derived as described and seeded into 24-well plates at 200,000 cells/well in antibiotic-free medium [25]. The next day, UPEC was added at the indicated multiplicity of infection (MOI) and the plate was centrifuged 750 g for 5 min to allow for efficient UPEC attachment. After 30 min at 37°C, the media was recovered and plated onto LB agar plates to enumerate extracellular CFU. To assess attachment, a separate set of UPEC-treated BMDM was washed with PBS, lysed with 0.1% Triton X-100 in PBS, and plated on LB agar [22]. To detect bacterial invasion, another set of UPEC-treated BMDM was incubated 30 min at 37°C and then washed three times with medium containing 100 μ g/mL gentamicin, which kills extracellular UPEC. Next, these BMDMs were incubated for additional

Fig. 1. *Rnase6*^{EGFP} reporter mice. **a** Schematic of the *Rnase6*^{EGFP} allele. See Methods and online supplementary Fig. S1 for additional details regarding the generation and initial evaluation of these mice. LA and SA: short and long arms. **b** *Rnase6* mRNA expression was quantified by QRT-PCR in WT adult female mouse tissues ($n = 4$ –8 mice/group). Relative *Rnase6* levels based on 2^{− $\Delta\Delta$ Ct} method are shown. Bars represent mean $\pm$ SEM for each

condition; **** $p < 0.0001$, ANOVA. **c** Representative scatterplots illustrate cellular sources of *Rnase6* in hematopoietic tissues, based on flow cytometric detection of EGFP fluorescence in *Rnase6*^{EGFP/+} mice (4 mice/condition for bone marrow and spleen; 6 mice/condition for peripheral blood; MFI values for EGFP fluorescence in each population are plotted in online suppl. Fig. S3). MFI, mean fluorescence intensity.



120 min in medium containing 20 µg/mL gentamicin, lysed with 0.1% Triton X-100 in PBS, and plated on LB agar to enumerate intracellular UPEC [36]. The percentage of extracellular, attached, and intracellular CFU was calculated by dividing the number of CFU recovered by the total number of CFU. The total number of CFU was measured by infecting BMDM with UPEC, incubating 30 min at 37°C, lysing with 0.1% Triton X-100 in PBS, and plating onto LB agar. Six replicate wells were evaluated for each experimental condition.

Macrophage Apoptosis and Necrosis

Rnase6^{+/+} and *Rnase6*^{EGFP/EGFP} BMDMs were incubated with UPEC (MOI 10) for 30 min and 3 h to investigate early apoptosis and late apoptosis/necrosis, respectively. Cells undergoing apoptosis and/or necrosis were identified by flow cytometry based on Annexin V and 7-AAD positivity as described [25].

Macrophage Phagocytosis

Overall, 200,000 *Rnase6*^{+/+} and *Rnase6*^{EGFP/EGFP} BMDMs were cultured in black, clear bottom 96-well plates, in RPMI-1640 medium supplemented with 10% fetal bovine serum. After 30 min of culture at 37°C in 5% CO₂, BMDMs were stimulated with fluorescein-labeled *E. coli* K-12 bioparticles (Vybrant™ Phagocytosis Assay Kit; Invitrogen) following the manufacturer's protocol. After 15, 30, and 60 min, the cells were fixed using 4% PFA. The cells were washed twice with PBS and analyzed by fluorimetry using a SpectraMax M2 plate reader with an excitation wavelength of 480 nm and emission wavelength of 520 nm. We confirmed that *Rnase6*^{EGFP/EGFP} cells had no background fluorescence under these conditions, justifying use of this method and noninterference by EGFP.

Macrophage Cytokine Elicitation

BMDMs from *Rnase6*^{+/+} and *Rnase6*^{EGFP/EGFP} mice were cultured in DMEM F12 medium supplemented with 10% fetal bovine serum and challenged with UPEC (MOI 10) for 24 h. Gentamicin (20 ng/mL) was added 30 min after inoculation to limit cytotoxicity. The supernatants were collected and stored at -80°C. Cytokine production was measured by electrochemiluminescence using a MESO QuickPlex SQ 120MM device (Meso Scale Discovery, Rockville, MD, USA) and the U-PLEX Biomarker Group 1 plate.

Statistics

Statistical analysis was obtained using Prism (GraphPad Software, La Jolla, CA, USA). Continuous data were assessed for normal distribution by the D'Agostino-Pearson omnibus test.

Normally distributed data were evaluated by Student's *t* test; the Mann-Whitney U test was used when comparing nonparametric data. Only *p* values <0.05 were considered significant.

Results

Generation of *Rnase6*^{EGFP} Knock-In Mice

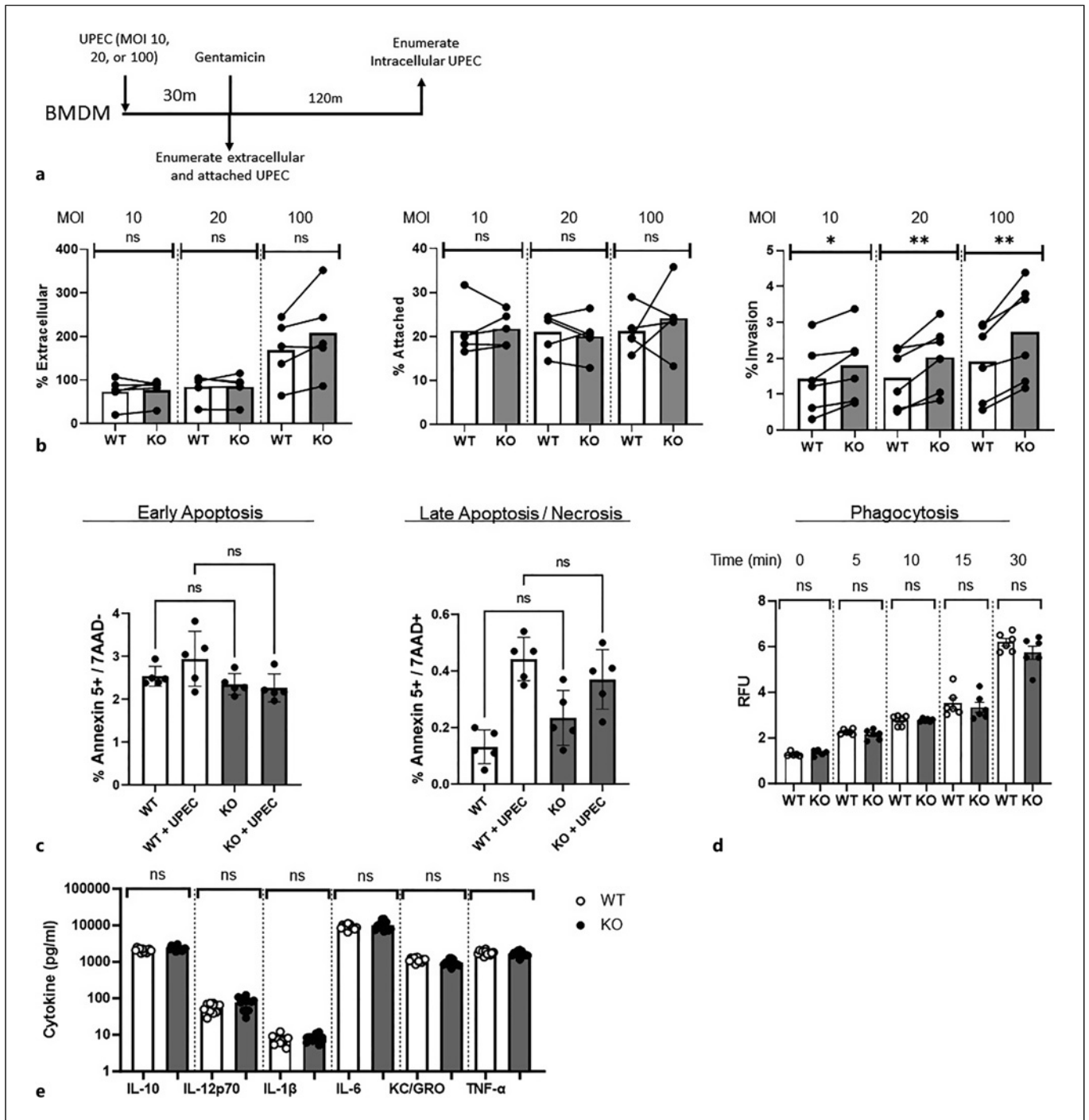
To study *Rnase6* expression and determine the consequences of homozygous *Rnase6* deletion on host antimicrobial activity toward UPEC, we engineered mice with an *Rnase6*^{EGFP} knock-in allele. This mouse strain, which exists on a pure C57BL/6J background, served as an EGFP reporter allele to identify cellular sources of RNase 6, as well as to function as an *Rnase6* null allele to determine the contributions of RNase 6 during experimental UTI when bred to homozygosity (Fig. 1a and online suppl. Fig. S1; for all online suppl. material, see <https://doi.org/10.1159/000539177>). We performed flow cytometry in adult *Rnase6*^{EGFP/+} mice to identify cellular sources of RNase 6. We initially focused on hematopoietic tissues, where *Rnase6* mRNA levels are most abundant (Fig. 1b) [37]. Using the cytometric gating strategy shown in online supplementary Figure S2, we detected EGFP fluorescence in monocytes (CD45⁺/CD11b⁺/Ly6G⁻/Ly6C^{+/-}) and macrophages (CD45⁺/CD11b⁺/Ly6C⁻/MHC-II⁺), but EGFP fluorescence was absent in neutrophils (CD45⁺/CD11b⁺/Ly6G⁺), of peripheral blood, spleen, and bone marrow from uninfected mice (Fig. 1c and online suppl. Fig. S3).

Monocytes and Macrophages Are Cellular Sources of RNase 6 during UTI

In the uninfected *Rnase6*^{EGFP/+} bladder, we detected EGFP fluorescence in resident macrophages and monocytes (Fig. 2a and online suppl. Fig. S3). Following transurethral UPEC inoculation, an influx of EGFP⁺ monocytes was detectable 24 h post-infection (hpi), consistent with published studies regarding monocyte recruitment during cystitis [38, 39]. We also observed

Fig. 2. Cellular sources of *Rnase6* and their localization within the urinary tract at baseline and following UPEC infection. **a** Flow cytometry implicates monocytes and macrophages as the principal cellular sources of mouse *Rnase6* expression in uninfected and UPEC-infected *Rnase6*^{EGFP/+} bladders and kidneys 24 hpi. The upper panel shows representative flow cytometry scatterplots of monocytes, macrophages, and neutrophils when gating on live CD45⁺CD11b⁺ cells at baseline or 24 h post-UPEC inoculation (hpi). The histograms below each scatterplot indicate fluorescence intensity of EGFP for each of the specified cell subtypes. Representative scatterplots from 6 mice are shown. Gating strategy and

MFI values for EGFP fluorescence in each population are shown in online supplementary Figures S2 and S3, respectively. **b** Distribution of EGFP (green) in the urinary tract of *Rnase6*^{EGFP/+} mice at baseline and 24 hpi. Representative ×40 images from *n* = 4–6 mice are shown. Urothelial cells and renal tubular epithelial cells are identified based on Krt7 reactivity (white). The area within each yellow inset is featured below each image. Dashed lines indicate the urothelial basement membrane. White arrows indicate submucosal EGFP⁺ cells. Yellow carets ^ indicate intraurothelial EGFP⁺ cells. See online supplementary Figures S5 and S6 for additional details. Krt7, Keratin 7; MFI, mean fluorescence intensity; U, urinary space.



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increased numbers of EGFP⁺ macrophages in infected bladders (Fig. 2a and online suppl. Fig. S4). While UTI led to robust neutrophil recruitment, we did not detect EGFP⁺ neutrophils (Fig. 2a). We observed similar expression of EGFP in monocytes and macrophages at

baseline and following UPEC infection in *Rnase6*^{EGFP/+} kidneys (Fig. 2a, online suppl. Fig. S3 and S4) [25].

To localize *Rnase6*⁺ cells in the urinary tract, we performed immunofluorescence microscopy with an EGFP antibody in *Rnase6*^{EGFP/+} mice (Fig. 2b). In

uninfected bladders, EGFP localized almost exclusively within the submucosa. Following UPEC inoculation, certain EGFP⁺ cells redistributed to the basal layer of the urothelium, though the majority remained in the submucosa (Fig. 2b and online suppl. Fig. S5). In the uninfected and infected kidney, EGFP⁺ cells were predominantly visualized in the perivascular space of the renal medulla, near or directly within the urothelium (Fig. 2b). To identify cellular sources of *Rnase6*, we generated *Rnase6*^{EGFP/+}/*Ccr2*^{RFP/+} reporter mice, in which *Ccr2*⁺ monocytes and monocyte-derived macrophages express the RFP [40]. We identified EGFP⁺/RFP⁺ cells in bladders and kidneys at baseline and following infection (online suppl. Fig. S6). These findings, which are consistent with observations using flow cytometry (Fig. 2a), confirm that monocytes and monocyte-derived macrophages are the primary sources of *Rnase6* in the urinary tract.

Rnase6-Deficient Mice Are Viable and Healthy, and Do Not Exhibit a Detectable Baseline Phenotype or Deficit

To determine the impact of *Rnase6* deficiency on UTI susceptibility, we generated *Rnase6*^{EGFP/EGFP} mice, which we refer to as *Rnase6* knockout (*Rnase6* KO). Adult *Rnase6* KO mice displayed no apparent phenotype at baseline including comparable weights, organ histology, serum chemistries, and complete blood counts and leukocyte differentials when compared to age- and sex-matched *Rnase6*^{+/+} C57BL/6J controls (online suppl. Table S1).

Rnase6 KO Macrophages Exhibit Impaired Intracellular UPEC Killing

Since monocyte/macrophages are the predominant cellular source of RNase 6 in the urinary tract during experimental UTI, we investigated the impact of

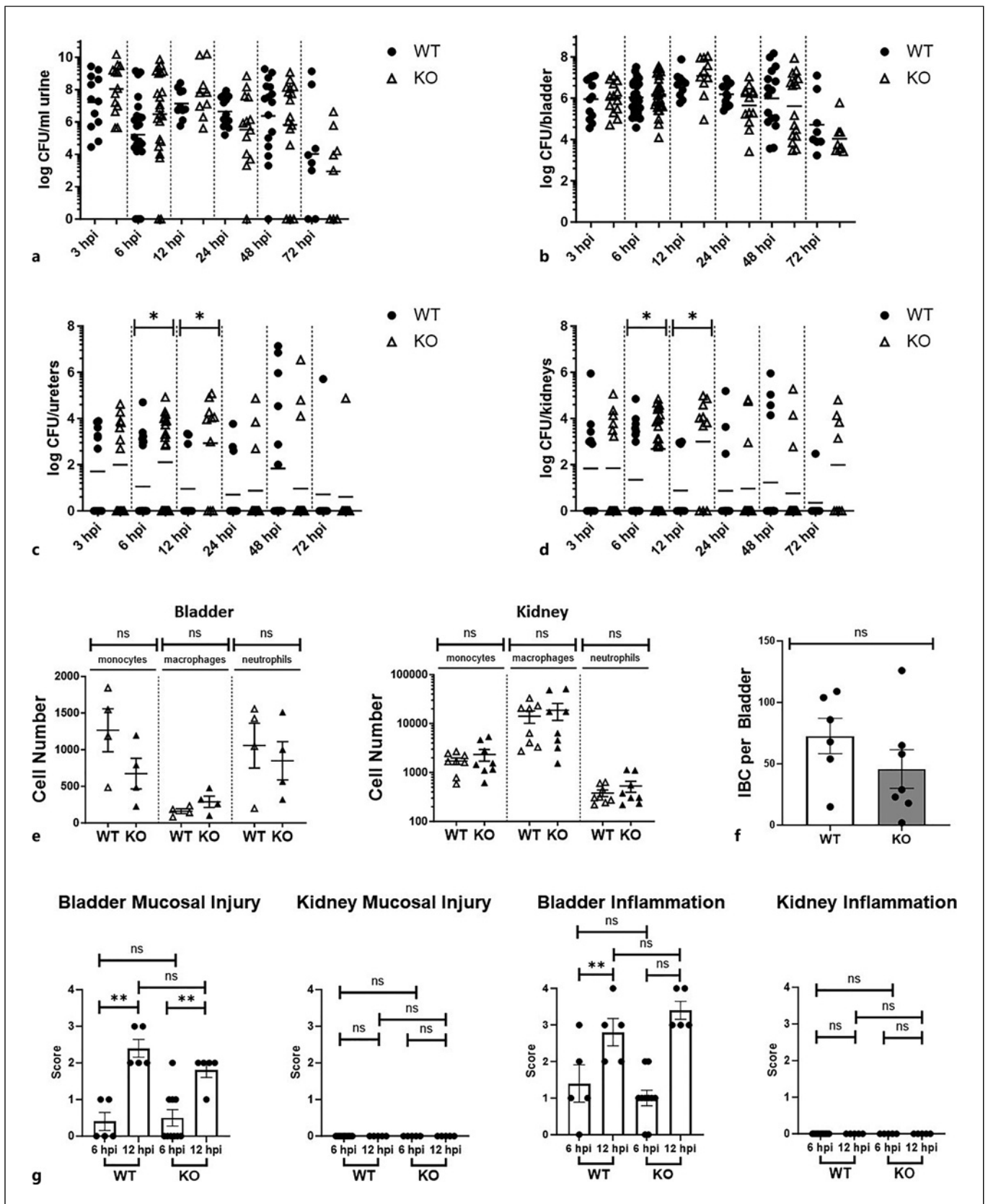
Rnase6 deficiency on UPEC killing by BMDMs. Equal numbers of *Rnase6* KO and WT control BMDM were inoculated with UPEC at varying MOI, and extracellular, attached, and intracellular UPEC was enumerated (Fig. 3a). We recovered significantly higher CFU of intracellular UPEC from *Rnase6* KO BMDM compared to WT BMDM at all MOI tested (Fig. 3b). In contrast, we did not detect differences in extracellular killing and attachment of UPEC between genotypes (Fig. 3b). This is consistent with the hypothesis that RNase 6 is required for optimal intracellular UPEC killing in macrophages. *Rnase6* KO and WT BMDM did not differ in their viability, phagocytic activity, or cytokine elicitation in response to UPEC (Fig. 3c–e).

Rnase6 KO Mice Are More Susceptible to UPEC Infection

Next, we subjected adult *Rnase6* KO and *Rnase6*^{+/+} C57BL/6J control females (WT) to experimental UTI and evaluated UPEC burden post-infection. We recovered significantly more UPEC from *Rnase6* KO ureters and kidneys 6 hpi and 12 hpi compared to WT controls (Fig. 4c, d). In contrast, there was no impact of *Rnase6* deficiency on urine or bladder UPEC burden, and upper tract UPEC burden was comparable between genotypes at subsequent time points (Fig. 4a–d). In addition, there was no impact of *Rnase6* deficiency on the absolute number of phagocytes in the bladder and kidney following UTI (Fig. 4e). *Rnase6* deficiency was not associated with alterations in mucosal injury, inflammation, or the number of intracellular bacterial communities (Fig. 4f, g). These studies establish that RNase 6 performs an essential early role in UPEC clearance from the upper urinary tract during ascending UTI.

Fig. 3. Impaired intracellular UPEC killing by *Rnase6* KO macrophages. **a** Experimental paradigm to test the contributions of RNase 6 to UPEC killing by BMDM. Time in minutes (m) is indicated. UPEC was added at the indicated MOI. After 30 min at 37°C, the media was recovered to enumerate extracellular CFU. To assess attachment, UPEC-treated BMDMs were washed, lysed, and CFU enumerated [22]. To detect bacterial invasion, UPEC-treated BMDMs were incubated 30 min at 37°C and then washed three times with medium containing gentamicin, which kills extracellular UPEC. Next, these BMDMs were incubated for an additional 120 min, lysed, and intracellular CFUs were enumerated [36]. The percentage of extracellular, attached, and intracellular CFU was calculated by dividing the number of CFU recovered by the total number of CFU. Further details are provided in Methods. **b** Increased recovery of intracellular UPEC from *Rnase6*^{EGFP/EGFP} BMDM (KO), compared to WT controls (6

independent experiments). In contrast, there was no impact of *Rnase6* deficiency on extracellular and attached UPEC (5 independent experiments). Each data point represents the average of 6 technical replicates from a single experiment. The bar designates the mean across experiments. Paired data are connected by lines and analyzed by paired *t* test (***p* < 0.01; **p* < 0.05; ns). **c** WT and *Rnase6* KO BMDM exhibit comparable levels of apoptosis and necrosis at baseline and following UPEC exposure (MOI 10) for 30 min (early apoptosis) and 3 h (late apoptosis/necrosis) (Mann-Whitney U test). **d** WT and *Rnase6* KO BMDM do not differ in their phagocytic capacity of fluorescein-labeled *E. coli* K-12 bioparticles (Mann-Whitney U test; RFUs; time after addition of bioparticles is indicated). **e** Similar magnitudes of cytokine elicitation by UPEC treatment of WT and *Rnase6* KO BMDM (Mann-Whitney U test). RFU, relative fluorescence unit; ns, not significant.



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Discussion

While mammalian genomes encode hundreds of AMPs [41], their cellular sources and functional significance remain largely untested in vivo. In a similar vein, RNase 6 is a highly evolutionarily conserved AMP with potent antimicrobial activity toward UPEC [23, 24, 26], but its physiological roles have remained uncertain. Accordingly, the dual purposes of this study were to (1) definitively establish the cellular sources of mouse *Rnase6* in vivo and (2) test the hypothesis that *Rnase6* serves an essential role in conferring antimicrobial activity toward UPEC. Our results indicate that RNase 6 is synthesized by monocyte/macrophages in the urinary tract and that *Rnase6* deficiency leads to increased susceptibility to ascending UTI. Our experiments in BMDM suggest that in vivo susceptibility to UPEC may arise as consequence of impaired intracellular UPEC killing by macrophages. Altogether, our findings demonstrate that *Rnase6* is essential for regulating acute UPEC infections in the upper urinary tract, potentially by facilitating macrophage-mediated antimicrobial mechanisms.

The use of the *Rnase6*^{EGFP} allele in this study provided an opportunity to definitively identify monocytes and macrophages as the primary cellular sources of *Rnase6* in the urinary tract and to track changes in their absolute number and localization during experimental UTI. Following UTI, we observed an influx of *Rnase6*^{EGFP}/Ly6C^{high} monocytes into the bladder, consistent with our findings in human *RNASE6* transgenic mice [37]. Similarly, we found that *Rnase6*⁺ cells translocated from the bladder submucosa to the urothelium following UPEC inoculation, a finding that parallels observations in *RNASE6* transgenic animals [37]. In the kidney, *Rnase6*^{EGFP}/+ reporter mice identified EGFP⁺ cells in the medulla, localizing to perivascular stroma and renal urothelium near the urinary space. This location, along with the observation of impaired intracellular UPEC killing by *Rnase6* KO macrophages, is consistent with a model in which renal macrophages phagocytose ascending UPEC and subsequently utilize *RNase6* to kill

intracellular bacteria. While intracellular UPEC killing is impaired in *Rnase6* KO BMDM, we did not observe differences in extracellular killing or UPEC attachment. These observations mirror those in *RNASE6* transgenic BMDM, which exhibited augmented intracellular UPEC killing when compared to non-transgenic controls, yet extracellular killing and UPEC attachment were unaffected. Both gain and loss of function data may be explained by the finding that *RNase6* localizes to the lysosome [42], where it is well situated to kill intracellular UPEC following phagocytosis by macrophages.

Despite the impact of *Rnase6* deficiency on antimicrobial activity of BMDM, *Rnase6* KO mice exhibited a relatively limited window of susceptibility to ascending UTI. The lack of a more overt UTI susceptibility phenotype in *Rnase6* KO mice was surprising to us, given the potent activity of RNase 6 peptide in vitro. One plausible explanation is that RNase 6 is functionally redundant with other RNase A family homologues such that loss of one RNase A member is insufficient to confer broader UTI susceptibility in vivo [15, 43–46]. Along these lines, *Ear11*, *Ear2*, and *Rnase4* encode RNase A members that are expressed by monocytes and macrophages and exhibit bactericidal activity toward Gram-negative bacteria [43, 46, 47]. It is possible that one or more of these RNases may exhibit functional redundancy with RNase 6 or altered expression in *Rnase6*-deficient mice. Another potential explanation is that *Rnase6*-deficient mice were developed on a C57BL/6J background that lacks vesicoureteral reflux and exhibits limited susceptibility to ascending UTI. Further experiments in C3H/HeOuJ mice with vesicoureteral reflux and an intact innate immune response are warranted to investigate the broader impact of *Rnase6* deficiency on susceptibility to PN [29, 30, 34].

Nonetheless, this study provides the first loss of function approach to implicate *Rnase6* in host defense against ascending UTI, prompting us to envision a model in which monocyte- and macrophage-derived RNase 6 serves an important role in limiting UPEC dissemination during PN. Our findings also provoke the hypothesis that strategies that augment *RNase6* levels or activity may be

Fig. 4. Increased susceptibility to ascending UTI in *Rnase6* KO mice. **a–d** Evaluation of UPEC burden in urinary tract tissues from *Rnase6* KO mice versus controls (WT): urine (**a**), bladder (**b**), ureters (**c**), and kidneys (**d**). Each point represents a single mouse. The horizontal line indicates the geometric mean. Equal numbers of mice of each genotype were analyzed at each time as follows: 13 (3 hpi), 24 (6 hpi), 10 (12 hpi), 13 (24 hpi), 16 (48 hpi), and 8 (72 hpi). **p* < 0.05, Mann-Whitney U test. **e** *Rnase6* deficiency does not impact the absolute numbers of monocytes, macrophages, and

neutrophils in bladders and kidneys 6 hpi (ns, Mann-Whitney U test). **f** Similar IBC counts in WT and KO bladders 6 hpi (average ± SEM is shown; *n* = 6–7/group; ns, Mann-Whitney U test). **g** *Rnase6* deficiency does not impact bladder or kidney histopathology during UTI (*n* = 5/group). Each point represents the bladder or kidney injury score from a single WT or KO mouse. The bar represents the average score, and error bars depict SEM ***p* < 0.01, Mann-Whitney U test. IBC, intracellular bacterial community; ns, not significant.

especially effective at limiting UPEC dissemination from the bladder and preventing PN in patients who are prone to UTI.

Acknowledgment

We thank Dr. Ailan Lu (InGenious Targeting Laboratory) for generating *Rnase6*^{EGFP/+} mice.

Statement of Ethics

This study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the Abigail Wexner Research Institute at Nationwide Children's Hospital (Protocol Number AR13-00057).

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

Funding Sources

This work was supported by the National Institutes of Health (NIDDK) R01 DK115737 and R01DK114035 (J.D.S.), R03 DK118306 (B.B.), K01 DK128379-01 (J.R.R.). We thank the *Agencia Estatal de Investigación* for the financial support to E.B. (PID2019-106123GB-I00/AEI/10.13039/501100011033).

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The Ohio State University Comparative Pathology and Mouse Phenotyping Shared Resource is supported by the National Institutes of Health (NCI) P30 CA016058. This project was supported by the Clinical and Translational Intramural Funding Program through the Abigail Wexner Research Institute at Nationwide Children's Hospital (Columbus, Ohio). Y.I.S.-Z. was supported by the Postdoctoral Idea Research Award at Nationwide Children's Hospital (Columbus, Ohio).

Author Contributions

Hanna Cortado and Juan de Dios Ruiz-Rosado designed and performed experiments, analyzed data, and prepared manuscript. Macie Kercsmar and Ashley R. Jackson: immunofluorescence microscopy. Biron Li performed experimental UTI and analyzed UPEC burden data. Gabriela Vasquez-Martinez and Diana Zepeda-Orozco: evaluation of cytokine production by BMDM. Sudipti Gupta and Christina Ching: enumeration of IBC during experimental cystitis. Gregory Ballash: evaluation of histopathology. Israel Cotzomi-Ortega and Yuriko I. Sanchez-Zamora: investigation of BMDM phagocytosis and viability. Ester Boix: data analysis and manuscript preparation. John David Spencer: experimental design and manuscript preparation. Brian Becknell: experimental design, data analysis, and manuscript preparation.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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