Supplementary Information for

Loss of Bladder Epithelium Induced by Cytolytic Mast Cell Granules

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Supplemental Data Inventory:

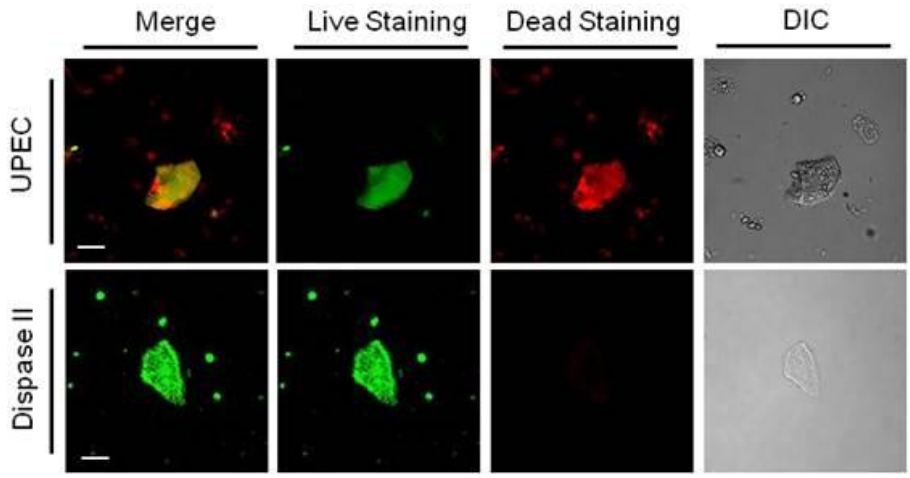

Figure S1, related to Figure 1. | In contrast to dispase-treated bladders, the majority of shed urothelial cells during bladder infection are nonviable. C56BL/6 mice bladders were inoculated with UPEC (upper panels) or treated with dispase II (lower panels), and urines were collected and cytospun to sediment BECs, which were subjected to live/dead staining. Dual staining by using ethidium homodimer-2 (red) and SYTO10 (green) were performed. Dead or dying BECs are indicated by positive staining of ethidium homodimer-2 and SYTO10 (upper panels). Live cells are detected by positive staining with SYTO10 (lower panels). Scale bars: 10 μm.

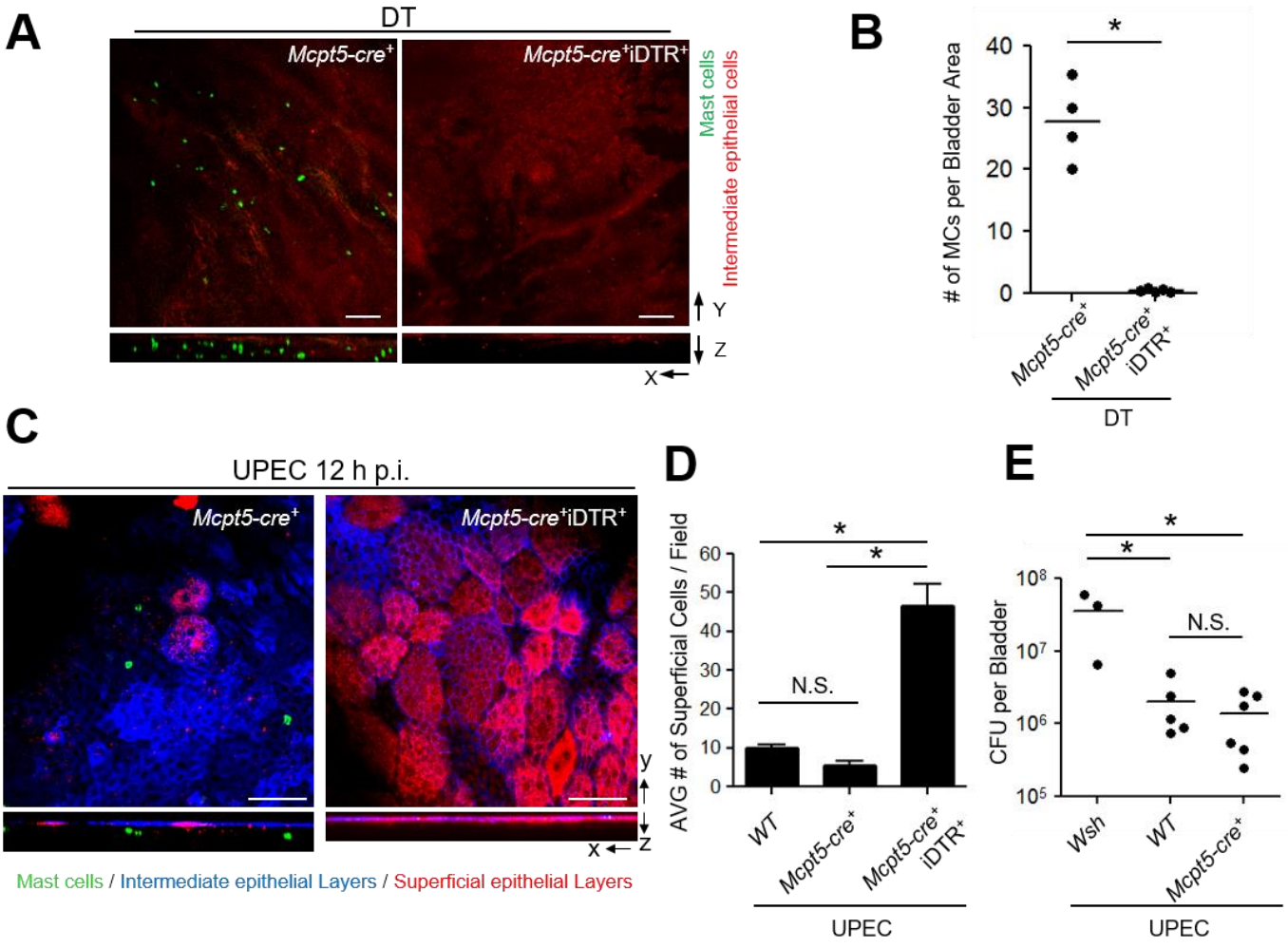
A
DT
Mcpt5-cre+
Mcpt5-cre+iDTR+
Mast cells
Intermediate epithelial cells
Y
Z
X
B
# of MCs per Bladder Area
40
30
20
10
0
*
Mcpt5-cre+
Mcpt5-cre+ iDTR+
DT
C
UPEC 12 h p.i.
Mcpt5-cre+
Mcpt5-cre+iDTR+
y
x
z
Mast cells / Intermediate epithelial Layers / Superficial epithelial Layers
D
AVG # of Superficial Cells / Field
60
50
40
30
20
10
0
*
*
N.S.
WT
Mcpt5-cre+
Mcpt5-cre+ iDTR+
UPEC
E
CFU per Bladder
10^8
10^7
10^6
10^5
*
*
N.S.
Wsh
WT
Mcpt5-cre+
UPEC
F
Isotype Control
1A8
CD11b
Ly6G
Neutrophils
3.93
Neutrophils
0.13
G
% population of Ly6G+ CD11b+ cells
5
4
3
2
1
0
*
Isotype Control
1A8
H
Isotype Control
1A8
Intermediate epithelial cells
Superficial epithelial cells
I
AVG # of Superficial Cells / Field
20
15
10
5
0
N.S.
Isotype Control
1A8

Figure S2, related to Figure 2. | Complete depletion of MCs in the bladder of *Mcpt5-cre*$^{+}$iDTR^{+} mice. **(A-E)** *Mcpt5-cre*$^{+}$ or *Mcpt5-cre*$^{+}$iDTR^{+} mice were injected *i.v.* with diphtheria (DT) to deplete MCs. The bladders from *Mcpt5-cre*$^{+}$ or *Mcpt5-cre*$^{+}$iDTR^{+} mice were dissected, stained for MCs with avidin (green) and for intermediate epithelial cells with α-E-cadherin antibody (red), and whole mounted for immunofluorescence microscopy. **Figure S2A** shows a compilation of projection images across different plane: X: axis, Y: Y axis, Z: Z axis, Notice that DT treatment of *Mcpt5-cre*$^{+}$*iDTR*$^{+}$ eliminated all of the MCs whereas DT treatment of *Mcpt5-cre*$^{+}$ mice had limited effect on MCs **(B)**. MC quantitation of randomly chosen fields in the two groups of mice were counted and compared. **(C, D)** MC dependent bladder exfoliation. *Mcpt5-cre*$^{+}$ or *Mcpt5-cre*$^{+}$iDTR^{+} mice were injected with DT to deplete MCs. Mice were infected intravesicularly. After 12 h p.i., mice bladders were dissected and whole-mount stained for imaging **(C)** and quantification **(D)**. **(C)** MCs (Avidin, green), intermediate epithelial cells (α-E-cadherin Ab, blue), and superficial epithelial cells (WGA, red). Heavy exfoliation of superficial epithelial cells occurred at *Mcpt5-cre*$^{+}$ mice bladder. In addition, MC degranulation at these mice reduced MC staining. On the other hand, we failed to see the MC population at *Mcpt5-cre*$^{+}$iDTR^{+} mice because of MC depletion, thereby lacking heavy exfoliation at superficial epithelial cells. N=3-5. **(E)** Lack of significant differences in CFUs between WT C57BL/6 and *Mcpt5-cre*$^{+}$ following UPEC infection. Wsh, WT, or *Mcpt5-cre*$^{+}$ mice were intravesicularly infected with UPEC. 12 h p.i., mice bladders were removed and CFU in this tissue measured. N=3-6. **(F, G)** Confirmation of neutrophil depletion with neutralizing antibody. Neutrophil neutralizing antibody (1A8) or isotype control antibody (2A3) were injected *i.p*. After 48 h, the blood of the mice was examined with flow cytometry analysis for neutrophil levels **(F)**. Approximately 95 % depletion was observed **(G)**. N=5. **(H, I)** Bladder exfoliation during UPEC infection is independent of neutrophil recruitment. Mice bladders obtained 12 h post-infection were stained for superficial epithelial cells (WGA, red) and intermediate epithelial cells (α-E-cadherin Ab, green) **(H)**. Notice that the loss of superficial epithelium in both groups are extensive but comparable **(I)**. Whole bladder tissues mounted onto slides for quantification. Ten random fields per bladder were imaged, and the number of superficial cells per field was enumerated. N=5. Scale bars: 100 μm**,** Error bar is SEM. *P<0.01, N.S.: not significant. **(B, G, I)** Two-way ANOVA for statistical individual comparison. **(D, E)** One-way ANOVA, Tukey's multiple comparison test.

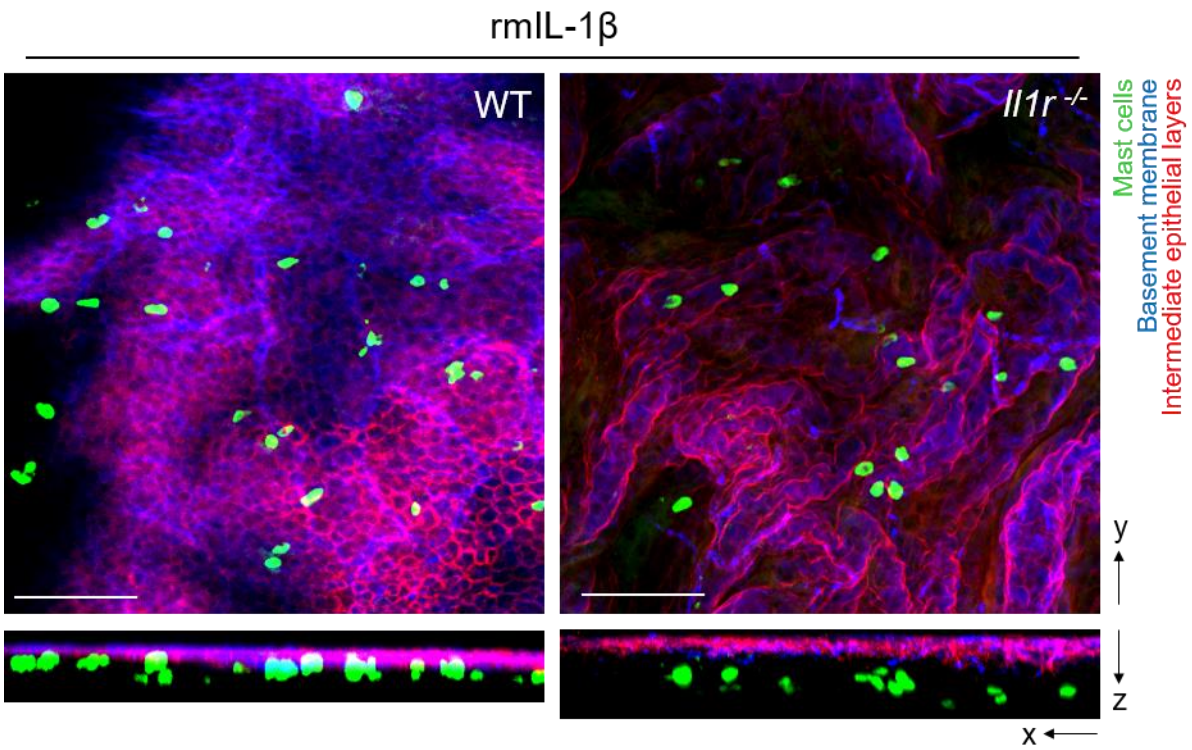

Figure S3, related to Figure 3. | MC recruitment by IL-1β in the bladder. WT or *Il1r*$^{-/-}$ mice were intravesicularly administered with rmIL-1β. After 4 h post-treatment, bladders were dissected and whole-mount prepared and stained for confocal imaging. MCs (Avidin, green), basement membrane (α-laminin Ab, blue), and intermediated epithelial layers (α-E-cadherin Ab, red). Z-stack images were deployed in XY and XZ planes.

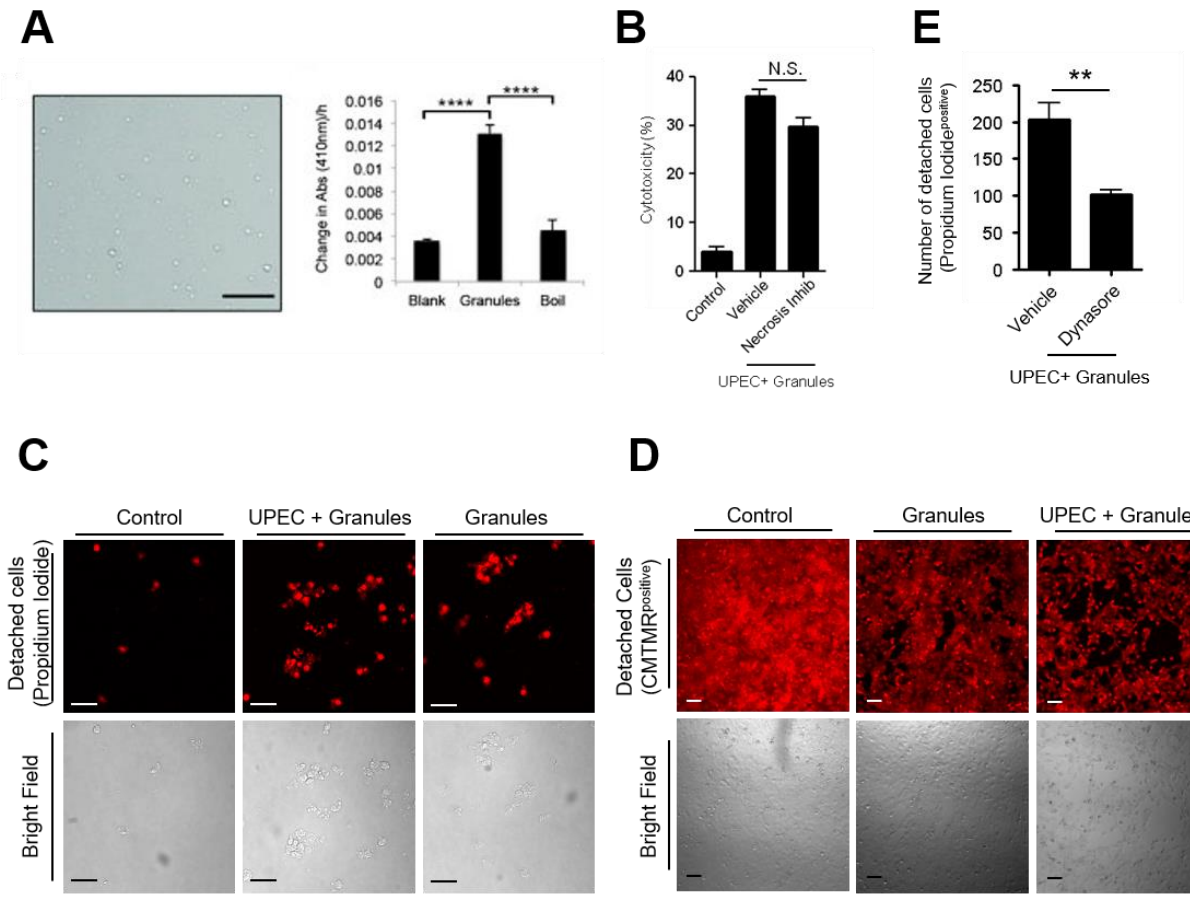

Figure S4, related to Figure 4. | (A) Isolated granules and the protease activity from granules. **(Left)** Isolated granules from the RBL-2H3 MC line. Scale bar indicates 25 µm. **(Right)** Biological activity of isolated MC granules confirmed in a tryptase activity assay. Tryptase activity was significantly higher in MC granule preps compared to blank controls or to boiled granules. Results are representative of 2 independent experiments and a total of n = 4 samples per group. **(B)** Necrosis inhibition fails to prevent MC granule-mediated cell death. 5637 BEC line were pre-exposed to a necrosis inhibitor (Necrox-2 at 20 µM) or vehicle. After 2 h, pre-treated cells were exposed to UPEC with MC granules. At 16 h post-treatment, LDH release was measured. **(C)** MC granules enhance the exfoliation of UPEC infected cells. Detached 5637 BEC line were stained with propidium iodide dye (top), and a corresponding bright field image is shown (bottom). **(D)** BEC shedding *in vitro* was enhanced when infected BECs were exposed to MC granules. The residual monolayers were stained with 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) live cell stain (top), and a corresponding bright field image is also shown (bottom). The black gaps in the monolayers in each of the panels reveal detachment of BECs. **(E)** Endocytosis inhibition significantly inhibits cell detachment and death. Uninfected and UPEC-infected 5637 BEC line were pre-treatment (30 min) with dynasore (100 µM) or vehicle and then exposed to MC granules. Granule treatment significantly enhanced death and detachment, which could be largely abrogated by dynasore treatment. Data representative of 3 independent experiments, n = 6 total samples per treatment. **(C, D)** Scale bars: 50 µm. Error bar is SEM. ****p<0.0001, **p<0.001, N.S.: not significant. **(A, B)** One-way ANOVA, Tukey's multiple comparison test. **(E)** Two-way ANOVA for statistical individual comparison.

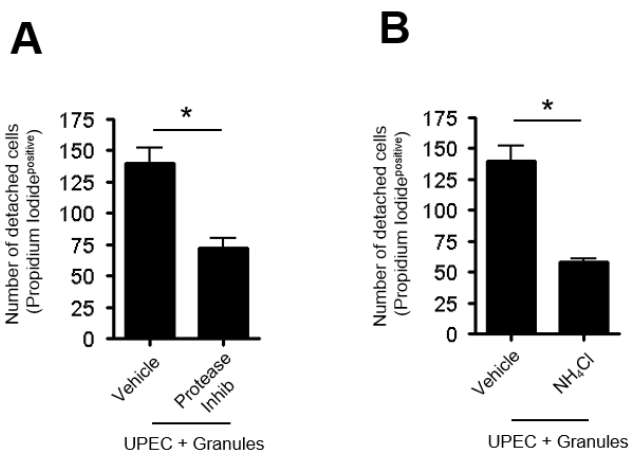

Figure S5, related to Figure 5. | (A) Inhibitors of proteases block granule induced detachment and cytoxicity in infected BECs. MC granules were pre-incubated with protease inhibitor (protease inhib) or vehicle for 30 min, and uninfected and UPEC-infected 5637 BEC line were exposed to MC granules. N = 6 total samples per treatment. Data representative of 3 independent experiments. **(B)** Inhibitors of endosome acidification block granule induced detachment and cytoxicity in infected BECs. Granules were added to pre-infected cells with or without pre-treatment (2 h) with NH_4Cl, a lysosome neutralizer. Detached cells were quantified following staining with propidium iodide to identify cell death. N = 6 total samples per treatment, Data representative of 3 independent experiments. Error bar is SEM. *$p<0.001$, Two-way ANOVA for statistical individual comparison.

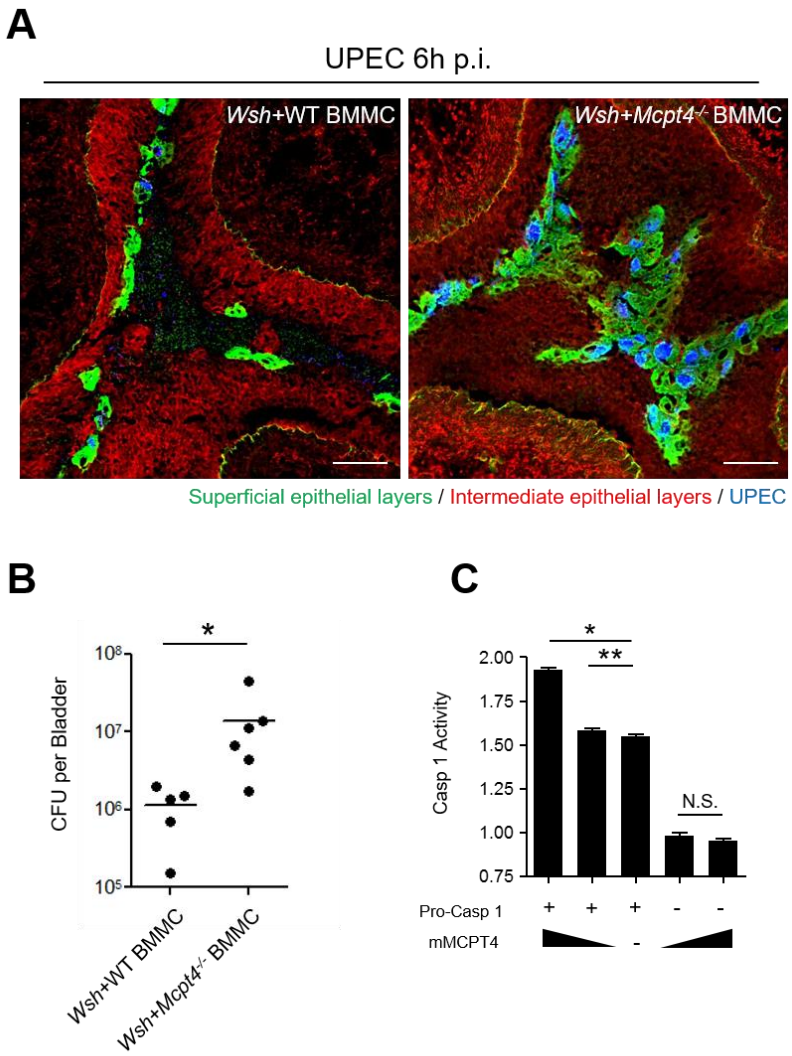

Figure S6, related to Figure 6. | mMCPT4 is critical to induce MC dependent bladder exfoliation during UPEC infection. *Wsh* mice were reconstituted with WT or *Mcpt4*$^{-/-}$ BMMCs. **(A, B)** The reconstituted mice were intravesicularly infected with UPEC. After 12 h post-infection, bladders were dissected for CFU or immunofluorescence imaging. WGA for superficial epithelial layers (green), α-E-cadherin antibody for intermediate epithelial layers (red), α-*E. coli* antibody for UPEC (blue) **(A)**. 5-6 mice were used for CFU **(B)**. Scale bars: 100 μm, **(C)** Assessment of caspase 1 activity. Caspase 1 substrate was added to the mixture of mMCPT4-TAT and pro-Caspase 1, and 1 h later the reaction was measured. Data represent 3 independent experiments. Error bar is SEM. *P<0.01, **P<0.05, N.S.: not significant. **(B)** Mann-Whitney test, **(C)** One-way ANOVA, Tukey's multiple comparison test.

Movie Legends

Movie 1, related to Figure 4. Real-time fluorescent movie showing the limited cell death in UPEC-infected BECs as labeled with propidium iodide on culture media.

Movie 2, related to Figure 4. Real-time fluorescent movie showing the pyroptosis in *Salmonella*-infected BECs as labeled with propidium iodide on culture media.

Movie 3, related to Figure 4. Real-time fluorescent movie showing the explosive cell death of UPEC-infected BECs after exposure to MC granules as labeled with propidium iodide on culture media.

Supplemental Experimental Procedures

Culture of BMMCs

Bone marrow was recovered from freshly sacrificed in ice-cold Hank's Balanced Salt Solution (HBSS), washed, and cultured in complete RPMI containing 10 % fetal bovine serum (FBS) (Sigma-Aldrich), 1 % HEPES (Gibco), 10 ng/ml SCF and 5 ng/ml IL-3 (BioLegend) for 8 to 12 weeks to obtain BMMCs. Cultures were maintained at a density of 10^6 cells/ml, and cells were fed every 2-3 days or passaged approximately once per week as needed.

***In vivo* infections**

Overnight cultures were inoculated from frozen stock in Luria broth (BD) and grown at 37˚C. To induce cystitis, mice were anesthetized with pentobarbital and catheterized with polyethylene tubing (inner diameter: 0.28 mm) (BD), and UPEC was instilled from a 1ml tuberculin syringe with a 30G1/2 needle. Following instillation, the catheters were removed, and mice were allowed to recover before sacrifice at various time points. Bladders were dissected from mice and immediately flash frozen in optimal cutting temperature (OCT) medium (Sakura Finetek). Or, for whole mount fluorescent imaging, bladders were excised, splayed open, and immediately fixed in 4% paraformaldehyde (PFA) at room temperature.

***In vitro* cell detachment assay, cytotoxicity and IL-1β ELISA assay**

$7x10^4$ cells were plated into 96-well cell culture plates. The next day, following growth to confluence on plates, 10 µg/ml mitomycin C (Sigma-Aldrich) was added to the fresh medium and incubated for 2.5 h. After washing, UPEC strain CI5 at MOI 100 was treated and spun at 300 x g. After 1 h of incubation, medium containing gentamicin (1 µg/ml) was used to wash and further incubated to kill extracellular bacteria. 100 µM dynasore (Sigma-Aldrich) for 30 min, protease inhibitors cocktail (10 µM aprotinin, 100 µM leupeptin, 100 µg/ml soybean trypsin inhibitor (SBTI), and 50 µM chymostatin (all from Sigma-Aldrich)) for 1 h or 100 mM NH_4Cl for 2 h were added to the media, prior to treatment with MC granules. After incubation for 16 h, detached cells were harvested from the culture supernatant and directly incubated with propidium iodide (10 µg/ml) (Molecular Probes) for 20 min to label nonviable cells. Detached cells were visualized via confocal microscopy, and the number of PI^+ cells per field was counted for 3 random fields from samples in triplicate. The remaining attached cells were stained with CMTMR dye (Molecular Probes) and visualized via confocal microscope.
To evaluate cytotoxicity, the percentage of total lactate dehydrogenase released into the supernatant was analyzed from the supernatant of samples collected at the indicated time points (Decker and Lohmann-Matthes, 1988).
To measure IL-1β secretion, enzyme-linked immunosorbent assay (ELISA) (R&D systems) was used.

MC granule isolation for *in vitro* studies

The rat MCs RBL-2H3 cells (ATCC) were cultured in MEM medium (Gibco) containing 10 % FBS and grown to near-confluence in 175 cm^2 cell culture flasks. After briefly rinsing with PBS, 1 µg/ml ionomycin (Sigma-Aldrich) in Tyrode's buffer was applied for 1-2 h to induce degranulation. The granule-containing supernatant and its PBS wash were removed, spun at 170 x g to separate out cell debris and then spun at 20,000 x g for 10 min to pellet granules. Granules from 3 T-175 flasks were combined and resuspended in 100 µl PBS and stored at -80°C until use. For *in vitro* detachment and cell death experiments, a 10 µl aliquot of granules was used per well in a 24-well plate. For *in vivo* studies with granules applied in mouse bladders, 30 µl granules were instilled.

Caspase-1 cleavage and activation assay

20 µg of mouse recombinant procaspase-1 were incubated with increasing amounts (0, 10, 20 µg) of recombinant mMCPT4 for 1 h at 37ºC in 27 mM Tris-HCl buffer containing 150 mM NaCl pH 7.4. Procaspase-1 cleavage was visualized by immunoblot using α-caspase-1 (p20) (Adipogen). mMCPT4 was detected by α-His_6 antibody (Roche). Caspase 1 assay kit (Abcam) was utilized to measure caspase-1 activation. The samples were mixed with 50 µM YVAD-AFC for 1 h at 37ºC in the reaction buffer. Fluorescence was measured at Ex/Em=400/505nm.

Mast cell migration assays

A Chemotaxis assay kit (Trevigen) was utilized as follows. Transwell membrane inserts (8.0 µm pore size) were coated at 37°C overnight with laminin I (200 µg/ml). BMMCs (5×10^4 cells/well) were suspended in serum reduced buffer (0.5% FBS) containing IL-3 ng/ml and plated onto the upper chamber. rmSCF or rmIL-1β (BioLegend) was added to the lower chamber of the transwell and the experimental system was incubated for 4 h at 37°C. Thereafter, the upper chamber was removed and the lower chamber was mixed with Calcein-AM solution and incubated for additional 30 min at 37°C. Fluorescence measured at Ex/Em=485/520 nm was employed to measure migrated MCs.

Immunofluorescent staining and microscopy

For frozen tissue samples, bladder tissue frozen in OCT compound were prepared as 12 µm sections by using a CM1850 Leica Biosystems cryostat (Leica Biosystems Inc); following this, the slides were fixed in ice-cold acetone, blocked in 1 % BSA-PBS, and incubated at 4 °C with primary antibodies against E-cadherin (BD Biosciences) or *E. coli* (Dr. Abraham laboratory) overnight. After washing, fluorescently-conjugated secondary antibodies (Jackson Immunoresearch) or fluorescently-conjugated wheat-germ-agglutinin

(Molecular Probes) were applied for 1 h at room temperature. Slides were mounted with Prolong Gold Antifade Reagent (Molecular Probes) for visualization with confocal microscope.

For whole mount bladders visualization, bladders were fixed for 2 h in 4 % PFA and blocked with buffer having 0.3 % Trion, 2.5 % normal goat serum (Gibco) in 1% BSA-PBS. Primary antibodies against ZO-1 (Invitrogen), laminin (Abcam) or E-cadherin (BD Biosciences) were applied overnight. Secondary α-fluorescently-conjugated antibodies (Jackson ImmunoResearch) or fluorescently-conjugated avidin (BD Biosciences) were incubated. Bladders were gently compressed and mounted on slides for confocal microscopy.

For imaging human BEC, 5637 cells were grown on glass cover slip and treated at described. 4 % PFA fixed cells and 0.1 % saponin in 1 % BSA-PBS solution permeabilized and blocked the samples. Primary antibody against α-LAMP1 antibody (Abcam) was used, and the secondary α-fluorescently-conjugated antibodies and phalloidin-Alexa647 (Invitrogen) were applied. After mounted on slides, the samples were visualized by confocal microscopy.

For *in vitro* live imaging, 5637 BECs were grown on MatTek plate (MatTek Corporation) and were infected with UPEC or applied with granules as described. After propidium iodide was applied to the media of cells, cells were placed under live cell station which is maintained at 37 ºC and 5 % CO2 with humidification. Zeiss Axio Observer microscope captured live moments.

α-mouse-FITC, α-rabbit-A647 or α-rat-Cy3 (Jackson ImmunoResearch) were used for secondary antibodies.

Supplemental Reference

Decker, T., and Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. Journal of immunological methods *115*, 61-69.