MC IL10 drive local tolerance in bladder infection

Supplemental Text:

The importance of MC-derived IL-10 was first demonstrated in a cutaneous sterile inflammation model, where MC-derived IL-10 was found to suppress subsequent inflammatory responses (Grimbaldeston, 2007). However, more recently, Dudeck *et al.* contested those findings and concluded from their studies using a novel inducible MC-deficient model that MC IL-10 was not relevant for suppression of contact hypersensitivity. Rather, the latter study concluded that the increased inflammation seen in Wsh MC-deficient mice was due to neutrophilia in Wsh mice as a result of the kit promoter mutation (Dudeck, 2011). To address this, we depleted neutrophils with an α-GR-1 monoclonal antibody (10ug/gram of mouse) 24 hours before urinary tract infection, in both WT and Wsh mice. Efficiency of neutrophil depletion was determined by FACS analysis of whole blood ($GR\text{-}1^{+}CD11b^{+}$) (**Figure S6A)** and myeloperoxidase assay for neutrophil recruitment to the bladder upon infection (**Figure S6B**). We saw that the absence of neutrophils did not change the increase of *Il10* expression seen in the bladder (**Figure S6C**) and the RLN still had significantly greater numbers of activated dendritic cells ($MHCII^{+}CD11c^{+}CD86^{+}$) as compared to the ILN (**Figure S6D**).

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Supplemental Figures

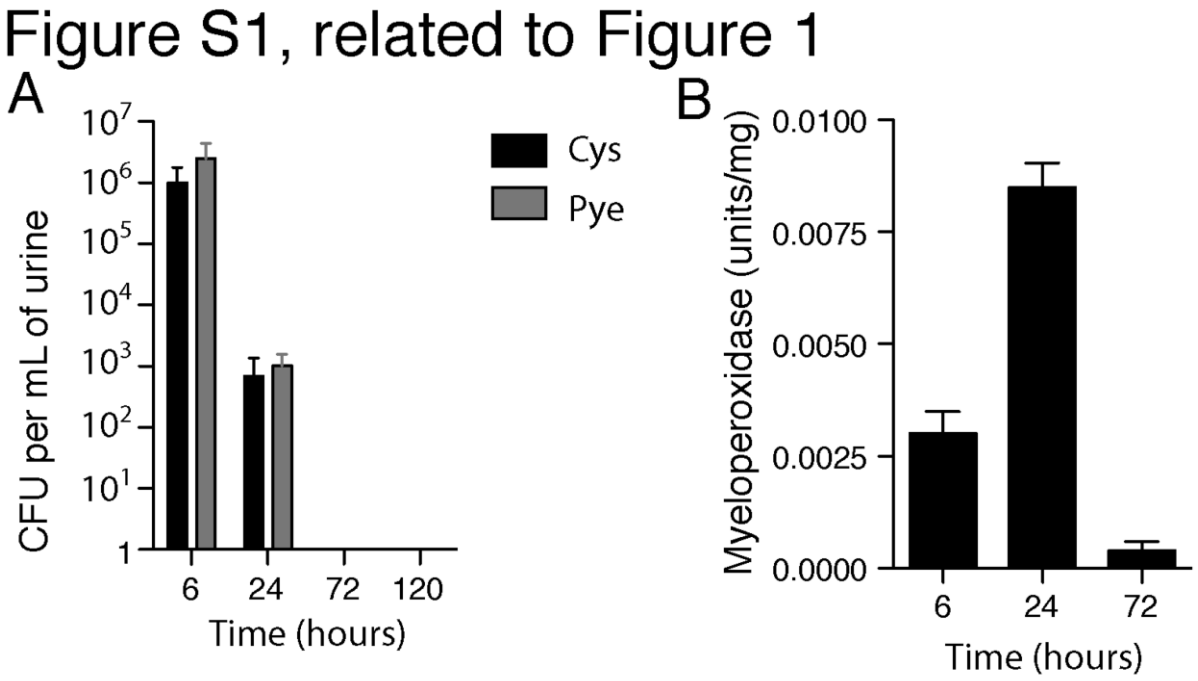

Figure S1, related to Figure 1. Rapid bacterial clearance in urine and neutrophil recruitment in bladders following infection. (A) WT mice were infected with either bladder-restricted or kidney infections and urine samples were examined for bacteriuria. No differences were seen between the kinetics of bacterial clearance in urine between the two models. Urine was sterile by day 3 post-infection. n = 3 mice per group; error bars represent ±SEM. **(B)** Recruitment of neutrophils is apparent in infected bladders as early as 6 hours post-infection, with peak numbers occurring at 24 hours, as determined by myeloperoxidase assay. At three days post-infection, decreases in neutrophils signify that acute inflammation is waning. All presented data are representative of three independent experiments with $n \geq 3$ for each time point. Error bars represent ±SEM.

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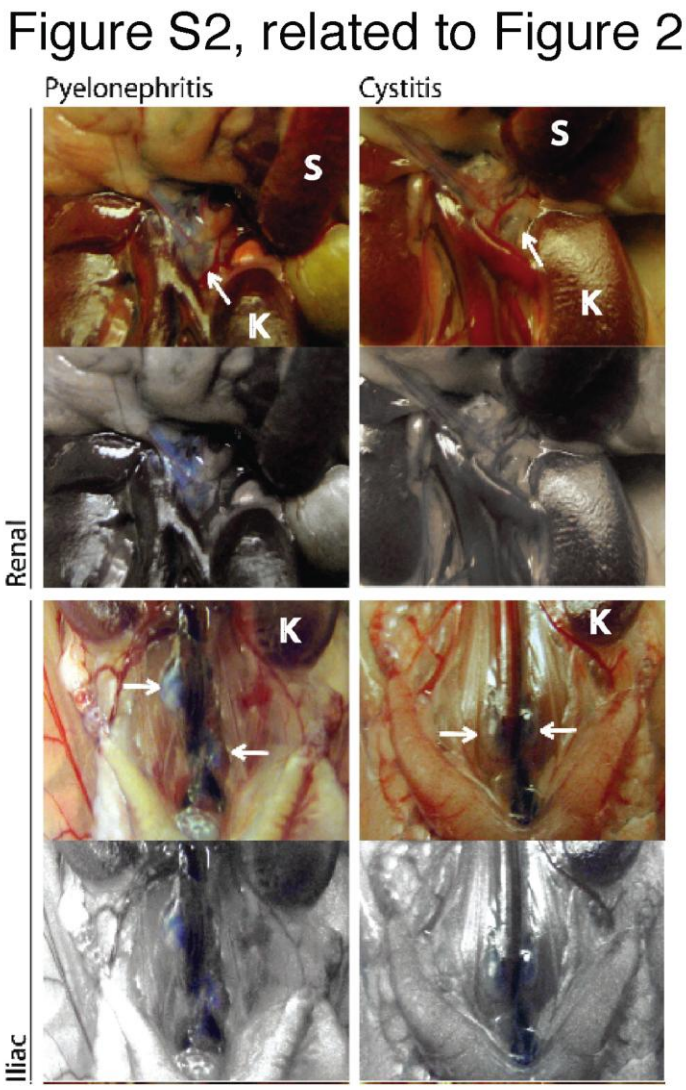

Figure S2, related to Figure 2. Murine Model of UTI distinguishing between pyelonephritis and cystitis. EBD is seen in both ILN and RLN (DLNs of the bladder and kidney, respectively) when mice were infected with induced-VUR, resulting in pyelonephritis (left panels). Note that EBD is seen only in the ILNs, and not in the RLN, with cystitis-only infection (right panels). Images in lower panels were processed to desaturate all colors except blue to facilitate visualization of EBD. Arrows denote the DLNs; K, the kidney; S, the spleen. In both models, mice were infected transurethrally with $1x10^8$ CFU of UPEC, mixed with EBD.

Figure S3, related to Figure 4

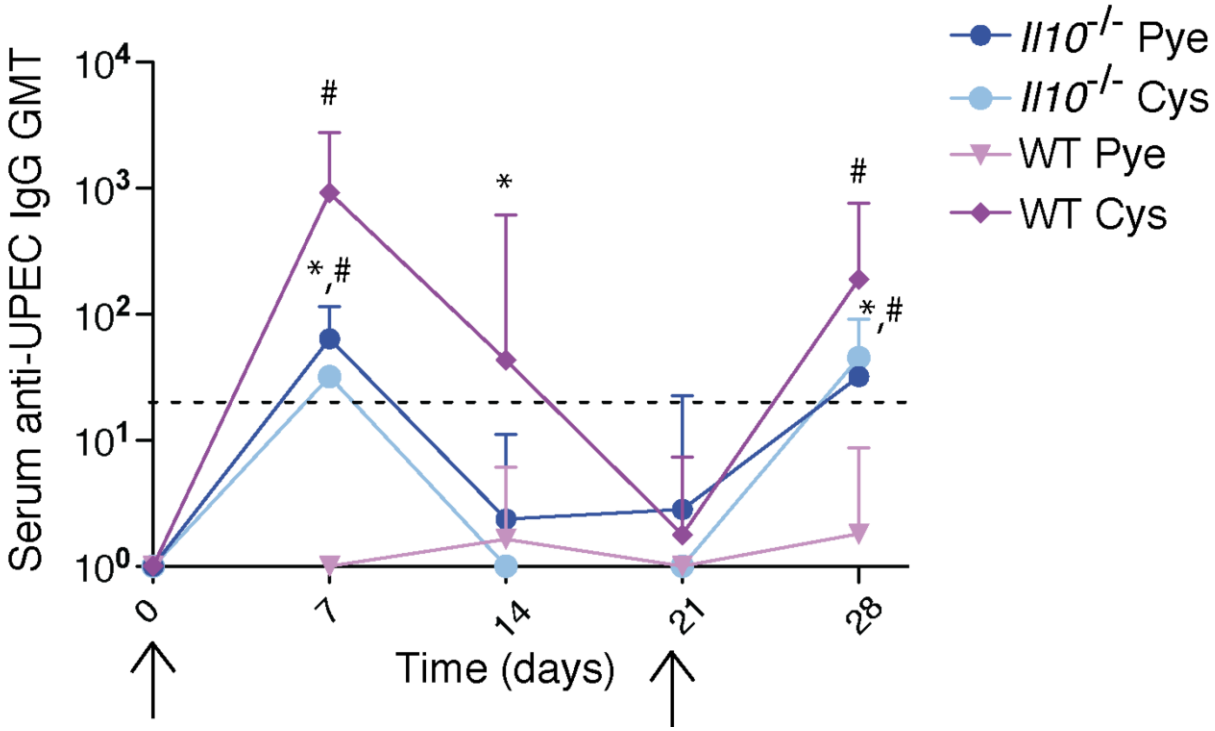

Figure S3, related to Figure 4. IL10$^{-/-}$ mice with cystitis-only infections have detectable anti-UPEC IgG antibodies in sera. IL10$^{-/-}$ were infected on day 0, and significant serum GMT anti-UPEC IgG antibodies are detected in both cystitis-only and mice with pyelonephritis in IL-10$^{-/-}$ mice. There were no significant differences in antibody titers between pyelonephritis and cystitis infected groups. On day 21 (second arrow) cystitis-only was induced in both groups of mice. *$p < 0.05$, # $p < 0.01$. Error bars represent the 95% confidence level with n = 5 mice per group.

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Figure S4, related to Figure 5

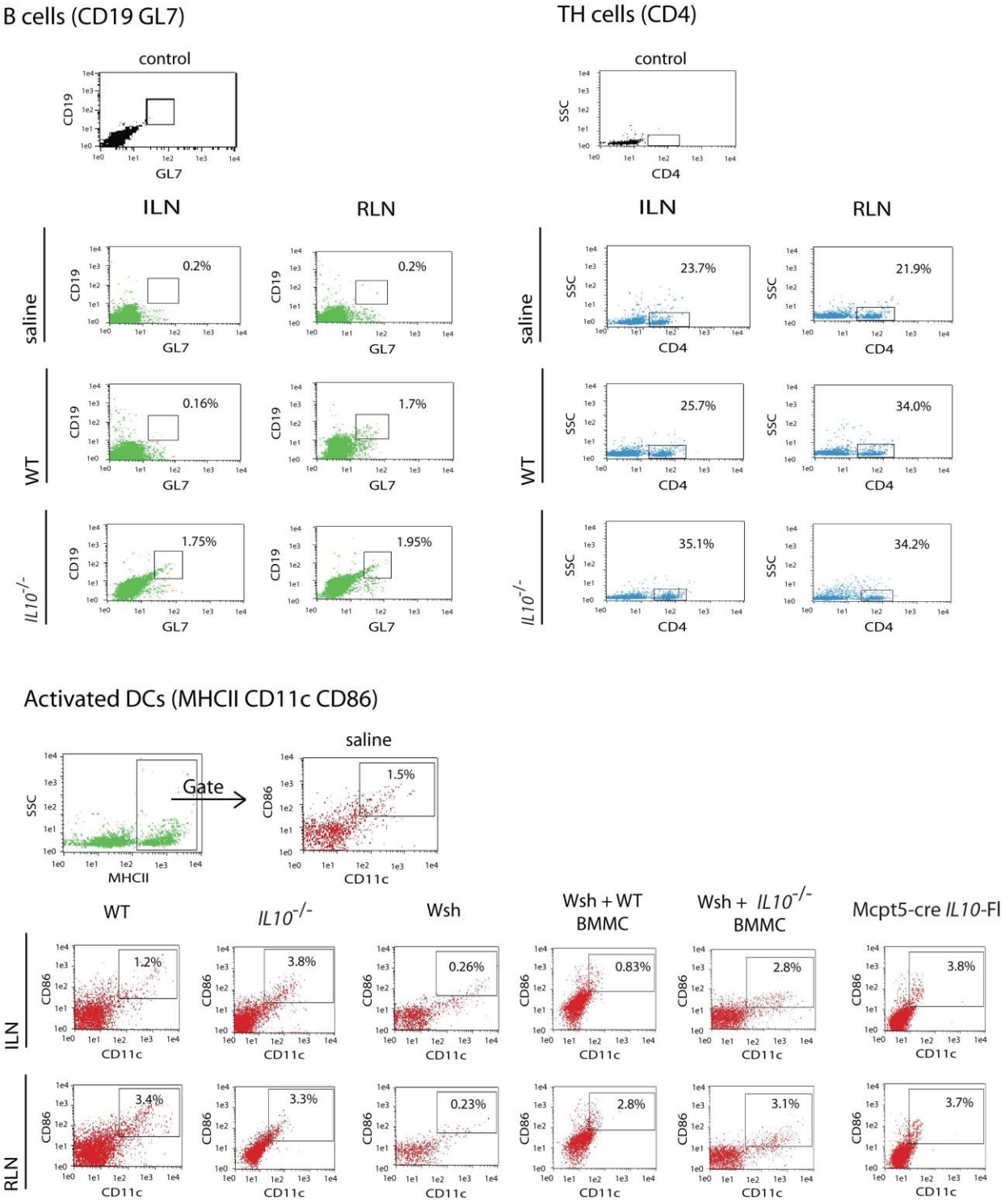

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Figure S4, related to Figure 5. Representative FACs plots of ILN and RLN. Mice were infected with either cystitis-only or pyelonephritis. DLNs were harvested 24 hours post-infection and made into single cell suspension for quantification of activated DCs ($MHCII^+CD11c^+CD86^+$) . For quantification and analysis of germinal center B cells ($GL7^+Cd19^+$) and T_H cells ($CD4^+$), DLNs were harvested at 7 days post-infection.

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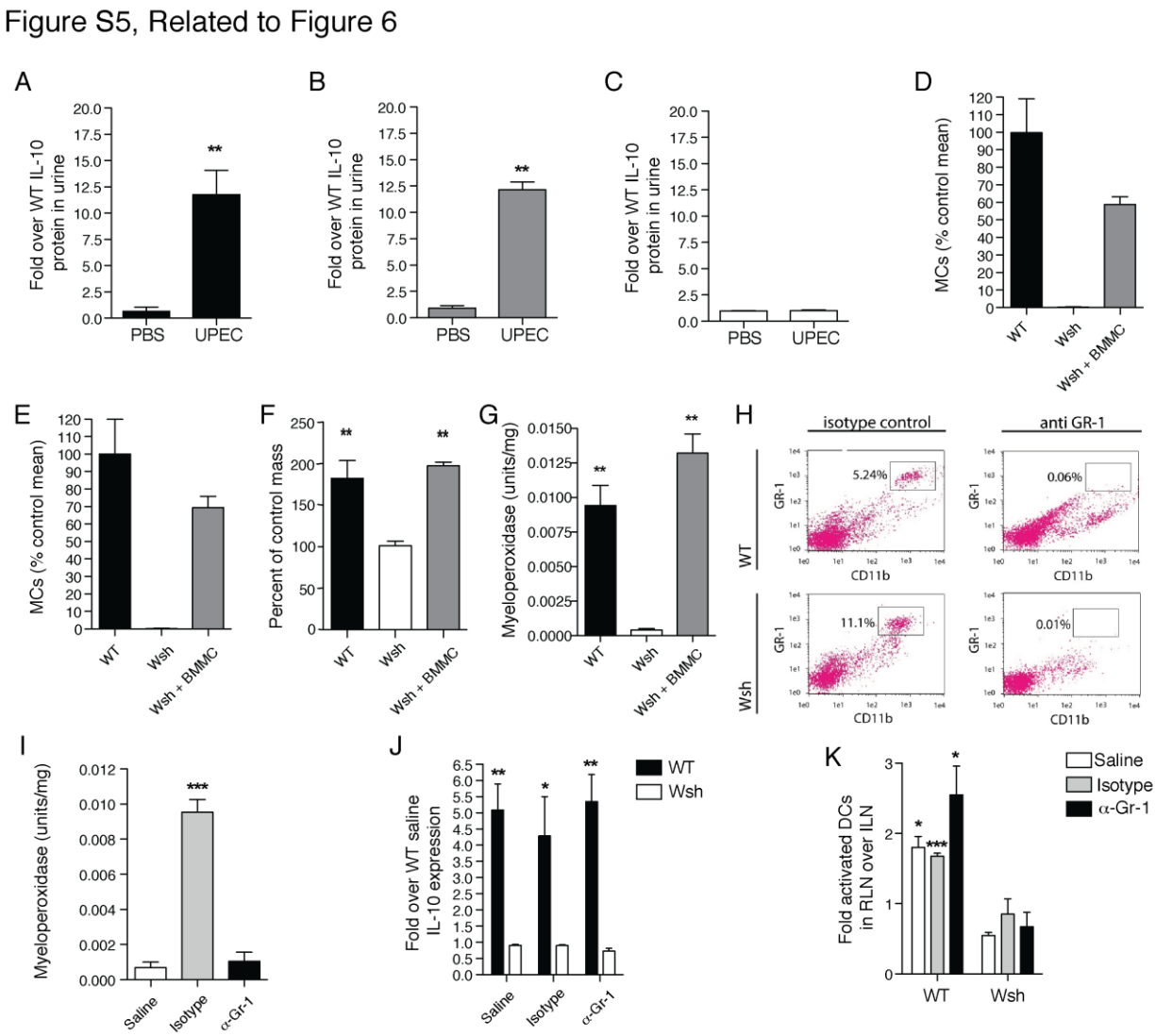

Figure S5, related to Figure 6. Difference in infection responses between infected WT and Wsh mice. WT, Wsh, and Wsh mice reconstituted with WT BMMCs were transurethrally infected with bladder-restricted infections. 24 hours post-infection, urine was sampled and the total amount of IL-10 protein in the urine was determined via a mouse IL-10 ELISA (ebioscience; Mouse IL-10 ELISA Ready-Set-Go; cat# 88-7104-22). Significant amounts of IL-10 are secreted and detected in the urine of UPEC infected **(A)** WT and **(B)** Wsh mice reconstituted with WT BMMCs but not **(C)** mast cell deficient Wsh mice. ** $p < 0.01$; n = 3-6

mice per group. Error bars represent ±SEM. Successful reconstitution of MC functionality within bladders of Wsh mice. Wsh mice were reconstituted with $1x10^7$ WT BMMC intravenously via tail vein. Fifteen weeks later, reconstituted Wsh mice were sacrificed and bladder and kidneys were harvested and snap frozen in optimal cutting temperature medium and stored at -80^oC until use. 20µm tissue sections were cut and fixed in Carnoy's fixative and stained with toluidine blue and mast cells were quantified through light microscopy. Mast cell numbers in **(D)** bladders and **(E)** kidneys of WT, Wsh and Wsh +BMMCs were compared. **(F)** The bladders of the three groups of mice were infected with UPEC and 24 hours after infection iliac LNs were examined for hypertrophy and **(G)** bladders were examined for neutrophil recruitment. We were able to see full recovery of WT phenotype in these reconstituted Wsh mice. $**p < 0.01$ Error bars represent ±SEM with n = 3-5 mice per group for all experiments. **(H)** Immunosuppressive response in infected bladders is not affected by neutrophilia phenotype of Wsh mice. WT and Wsh mice were pre-treated with an anti-GR-1 mAb or isotype control 24 hours before being infected. The efficiency of neutrophil depletion was determined by FACs analysis of whole blood and **(I)** neutrophil recruitment to the site of bladder infection via myeloperoxidase assay. **(J)** Neutrophil-depleted WT mice still have an increase of *Il10* expression in infected bladders 24 hours post-infection, which is not seen in neither Wsh nor neutrophil-depleted Wsh mice, suggesting that neutrophils do not influence the immunosuppressive microenvironment seen in the bladder upon infection. **(K)** Twenty-four hours post-infection, ILN and RLN were analyzed for draining of activated DCs ($MHCII^+CD11c^+CD86^+$). The draining of activated DCs into the RLN is significantly greater in WT mice as compared to the ILN, even with depletion of neturophils; this difference is not seen in infected Wsh mice. $* p < 0.05$, $** p \leq 0.01$, $***p \leq 0.001$; all data are

representative of three or more independent experiments totaling n = 3. Error bars represent ±SEM.

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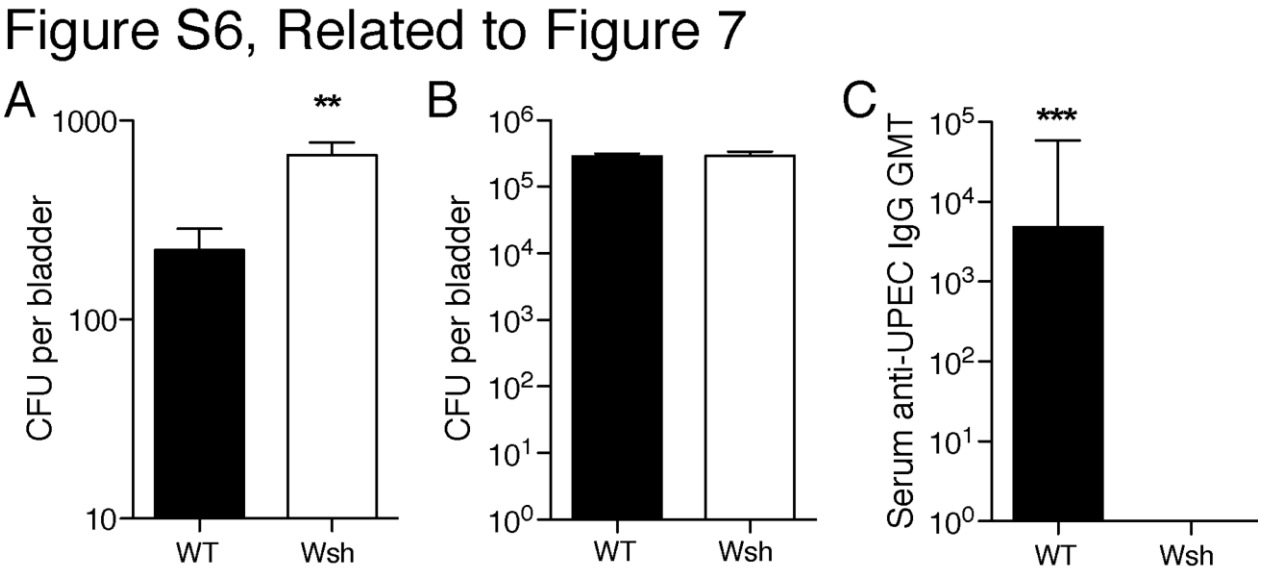

Figure S6, related to Figure 7. Mast Cells are Indispensible for Bacterial Clearance in the Bladder and Adaptive Immune Responses in Kidneys. **(A)** WT and Wsh mice were infected with $1x10^8$ CFU UPEC. Bladders were harvested 6 weeks after initial infection for quantification of residual persistent bacteria. Despite the contributions of MCs to bladder tolerance, their first and primary response is proinflammatory, and as a result, Wsh mice retain greater persistent bacterial numbers in their bladders. ***p* <0.01 Error bars represent ±SEM with n = 3-5 mice per group. **(B)** However, during initial infection - 4 hours - the intracellular bacterial burden does not differ between the bladders of WT and Wsh mice. Error bars represent ±SEM with n = 3-5 mice per group. **(C)** The anti-UPEC IgG GMT in Wsh mice is also significantly lower than that of WT mice with pyelonephritis. ****p* <0.01; Error bars represent the 95% confidence level with n = 5 mice per group.

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Supplemental Table

	sense	antisense
β-actin	5'-GAT-TAC-TGC-TCT-GGC-TCC-TAG-C-3'	5'-GAC-TCA-TCG-TAC-TCC-TGC-TTG-C-3'
IL-6	5'-ATC-CAG-TTG-CCT-TCT-TGG-GAC-TGA-3'	5'-TAA-GCC-TCC-GAC-TTG-TGA-AGT-GGT-3'
KC	5'-GCC-AAT-GAG-CTG-CGC-TGT-CAG-TGC-3'	5'-CTT-GGG-GAC-ACC-TTT-TAG-CAT-CTT-3'
IL-10	5'-GGT-TGC-CAA-GCC-TTA-TCG-GA-3'	5'-ACC-TGC-TCC-ACT-GCC-TTG-CT-3'

Real-Time PCR Primers

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Supplemental Methods

Bladder whole mount. Bladders were bisected and fixed in acetone, 2h, followed by overnight in PBS+1%BSA at 4^{o}C. Avidin-TRIC (Sigma-Aldrich) was added to the wells for incubation overnight at 4^{o}C and washed three times with the third wash at 4^{o}C overnight. Bisected whole bladder was spread out on a slide and coverslipped for visualization under laser-scanning confocal microscopy.

***CFU counts of persisting bacteria*.** To determine the numbers of bacteria, bladders and kidneys were aseptically harvested and homogenized in 0.1% Triton-X (Sigma) with zirconium oxide beads (GlenMills) by automatic homogenizer before plating on McConkey agar plates and incubating at 37^{o}C overnight.

RNA Isolation and real-time PCR. For total RNA isolations, bladders or kidneys were homogenized as earlier described and RNA was isolated using an RNeasy purification system (Qiagen), according to manufacturer's instructions. cDNA was synthesized with the iScript cDNA synthesis kit (BioRad) and we used SYBR Green and iCycler machine (BioRad) for real-time PCR. All target gene RNA expressions were normalized to actin expression. We obtained primers from IDT-DNA for *β-actin*, *Il6*, *KC*, and *Il10*. Primer sequences are provided in **Supplemental Table**.

In vivo neutrophil depletion. For studies requiring the depletion of neutrophils, WT and Wsh mice were intraperitoneally injected α-mouse GR-1 monoclonal antibody (10ug/gram of mouse)

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24h before induction of urinary tract infection. Efficiency of neutrophil depletion was determined via flow cytometry analysis of blood leukocytes and myeloperoxidase assay for neutrophil recruitment during cystitis.

Serology and ELISAs. C57BL/6 and *Il10*$^{-/-}$ mice were infected with either the slow or fast rate of bacterium inoculation as earlier described with *E. coli* CI5. Blood was drawn every 7d post-infection via the submandibular vein. An ELISA was used to monitor serum levels of anti-UPEC IgG end-point titers. Black 384 well ELISA plates (DYNEX) were coated overnight at 4^{o}C with 10^6 CI5 strain of UPEC in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$), blocked with carbonate buffer + 3% nonfat dry milk + 0.1% kathon for 2h at room temperature (RT), and incubated at 4^{o}C overnight with naïve or infected mouse sera diluted in complete sample diluent (PBS, 1% BSA, 1% non-fat dry milk, 5% normal goat serum, 0.05% Tween 20, and 0.1% kathon), with starting dilutions at 1:2^5 and serial diluting 2-fold across the plate. Plates were washed 4x with wash buffer (PBS, 0.05% Tween 20, 0.1% kathon) using an automatic 96-well plate washer (Packard Instrument Company) and alkaline phosphatase-conjugated mouse-IgG detection antibodies (Southern Biotechnology Associates) diluted in secondary antibody diluent (PBS, 0.05% BSA, 0.05% Tween 20, 0.1% kathon) was added. Plates were incubated at RT for 2h, washed 4 times with wash buffer and the fluorescent alkaline phosphatase substrate AttoPhose (Promega) was added for incubation at RT for 15 minutes in the dark. ELISA plates were read on a FluoroCount fluorescent plate reader (Packard Instrument Company). Samples were considered positive for antigen-specific antibody when the relative light units (RLU) reading for the sample dilution was 2 fold higher than the RLU for a naïve sample.

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Evans Blue Dye drainage to LNs. To visualize and confirm the technique of infection, Evans Blue dye (10mg/mL) was mixed in with the bacteria inoculum. Three hours post-infection, mice were sacrificed and the visual blue color changes of the DLNs were observed.

Adoptive transfer of splenocytes. Three weeks post-infection, spleens were aseptically harvested and processed into single cell suspensions. Spleens were minced in RMPI containing 10% FBS and 0.1mg/mL collagenase A (Sigma). EDTA (10mM, pH= 7) was added into each well after 60 min incubation at 37^{o}C. Single cell suspensions were produced by straining the distrupted spleens through a 0.7μm filter (BD Bioscience). Splenocytes ($2x10^{7}$, determined by hemacytometer counting) were transferred into naïve mice via tail vein injection.

Immunofluorescence and quantification of MCs in bladder sections. After 24h of infection, bladders were removed, snap frozen in optimal cutting temperature medium (TissueTek) and stored at -80^{o}C until use. 20-μm frozen sections of uninfected and infected bladders were cut and fixed in acetone for 15 minutes at 4^{o}C, blocked with 1% BSA for 1h at RT, and incubated with antibodies against mMCP6 (R&D, MAB3736) at 4^{o}C overnight. Sections were incubated with FITC-conjugated α–rat IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for 1h at RT, washed three times with PBS, and incubated overnight at 4^{o}C with APC-conjugated α-mIL10 (eBioscience). Coverslips were mounted using Prolong Gold anti-fade reagent (Molecular Probes) and viewed by confocal microscopy. For quantification of total numbers of MCs, each bladder was serial sectioned in its entirety 10-μm in thickness every 30-μm (approximately 120

sections per bladder). Sections were fixed in Carnoy's fixative for 15 minutes at RT and stained with toluidine blue. Total numbers of MCs were quantified via examination under light microscopy.

Supplemental References

Dudeck, A., Dudeck, J., Scholten, J., Petzold, A., Surianarayanan, S., Köhler, A., Peschke, K., Vöhringer, D., Waskow, C., Krieg, T., Müller, W., Waisman, A., Hartmann, K., Gunzer, M., Roers, A. (2011). Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity *34*, 973-984.

Grimbaldeston, M.A., Nakae, S., Kalesnikoff, J., Tsai, M., Galli, S.J. (2007). Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. Nat. Immunol. *8*, 1095-1104.