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Original Article

Estrogen receptor alpha differentially modulates host immunity in the bladder and kidney in response to urinary tract infection

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Abstract: The protective role of endogenous estrogen against Urinary Tract Infection (UTI) is well recognized, but the involvement of estrogen receptors (ERs) in modulating immunity in the urinary tract during UTI pathogenesis has not been investigated. The current study investigates the role of ERα in modulating immune responses and UTI outcome. Mice were pre-treated with either ERα agonist, propyl-pyrazole-triol (PPT), or ERα antagonist, methyl-piperidino-pyrazole (MPP), before experimental UTI. The UTI outcome was determined by checking the bacterial load, CD55 and TNFα expression in the bladder and kidney tissues. We observed opposite effects of PPT and MPP treatment on bacterial clearance in bladder versus kidney. PPT significantly reduced bacterial load *($P < 0.05$)* only in the kidney, with minimal changes in CD55 and TNFα levels. In contrast, MPP showed remarkable bacterial clearance only in the bladder that corresponded with reduced CD55 and TNFα expression. MPP treatment in uninfected state induced a significant increase in TNFα production *($P < 0.05$)* in the bladder, but not in the kidney. Our results suggest a protective role of ERα in the kidney. However, protection in the bladder may be mediated via other ER subtypes that may be involved in boosting the local immune responses. Drugs targeting specific ERs in bladder may serve as an adjunct treatment for boosting immune responses in the urogenital tract for efficient bacterial clearance.

Keywords: Estrogen receptor, urinary tract infection, bladder, kidney, urinary tract immunity, ERα agonist, ERα antagonist, CD55, TNFα

Introduction

Urinary Tract Infections (UTIs) are one of the most common bacterial infections, resulting in around one million hospital admissions in the United States annually [1]. Despite antibiotics being the most common regimen for UTI, they are becoming increasingly ineffective due to emergence of antibiotic-resistant microorganisms [2]. Women are more susceptible to UTI as 50-60% women experience at least one UTI episode in their lifetime and about 25% of these women have chances of acquiring recurrent UTI after the first infection. Postmenopausal women, who have sub-physiological levels of circulating estrogen, are more prone to acquiring recurrent UTI [3] which can often lead to acute pyelonephritis and kidney failure [3, 4]. Numerous other clinical reports and experimental studies on UTI have also indicated that estrogen is an important host factor in UTI pathogenesis [4-8]. Although, the FDA has approved the use of vaginal estrogen suppositories for post-menopausal UTI patients, the underlying mechanisms of action of these vaginal estrogen suppositories are not well understood [9].

About 80% of UTIs are caused by uropathogenic *Escherichia coli* (UPEC) like Dr fimbriae bearing *E. coli* (Dr *E. coli*), which cause both cystitis and pyelonephritis [1, 10]. By binding to its host receptor, CD55, which is also a complement regulatory protein [11], Dr *E. coli* internalize into bladder and kidney cells, forming intracellular bacterial reservoirs and leading to recurrent UTI [12, 13]. Studies have shown that estrogen impacts Dr *E. coli* binding in the human endometrium [14] and regulates the expression of CD55 in various human [14, 15] and mouse tissues [16]. Other reports have also demonstrated that estrogen treatment reduces UTI outcome in ovariectomized mice [6, 17] as well as in post-menopausal women confirming the protective role of estrogen [6]. However, the involvement of estrogen receptors (ERs) in generating immune responses in the urogenital tract against UTI has not been studied.

ER subtypes (ERα, ERβ and GRP30) are differentially expressed in various human and mouse tissues [18-24]. For example, several studies have reported a higher expression of ERα than ERβ in the kidney. The differential distribution of ERs in various tissues result in variable action of estrogen observed in these tissues [25]. ERs are known to induce or repress the transcription of numerous genes including early and late cytokine genes, thus playing a major role in regulating innate immune responses against infections [26-31]. While the immunomodulatory action of ERs have been studied in various viral [32] and bacterial infections [33, 34], their contribution in eliciting immune responses in the female urinary tract during UTI has not been investigated.

Innate immune responses in the urinary tract are robust and play a major role in UTI pathogenesis [2, 35]. As the first line of defense, mucosal epithelial cells are known to eliminate bacterial colonies by releasing pro-inflammatory cytokines like TNFα [36-38]. Studies have shown that TNFα levels in the urine samples of UTI patients were found to be considerably higher and reduced after therapy as compared to healthy individuals [39]. TNFα expression in various tissues has been found to be differentially regulated by different ER subtypes [40-44] under the influence of estrogen [45, 46]. Therefore, it is important to identify the specific ERs that are involved in TNFα production in the urinary tract in response to ascending UTI.

Results from our previous *in vitro* studies in mouse Inner Medullary Collecting Duct (mIMCD3) cells showed that activating ERα with the specific agonist, propyl-pyrazole-triol (PPT) [47], resulted in 50-60% reduction in bacterial invasion by Dr *E. coli*, while blocking ERα with the specific antagonist, methyl-piperidino-pyrazole (MPP) [48], reversed this protection by modulating CD55 expression (unpublished data). Based on our previous published study showing hormonal regulation of Dr *E. coli* colonization [14] and our results in mIMCD3 cells, we hypothesized that ERα is involved in dictating UTI pathogenesis by modulating CD55 and TNFα expression in the urinary tract. In the current study, we treated UTI susceptible C3H/HeJ ovariectomized (OVX) mice with PPT, and ovary-intact mice with MPP, before inducing experimental UTI. UTI outcome was determined by checking the bacterial load, CD55 and TNFα expression in both bladder and kidney. We observed opposite effects of PPT and MPP treatment on bacterial clearance and differential expression of CD55 and TNFα in bladder versus kidney. In conclusion, our results indicate that ERα is responsible for the bacterial clearance in the kidney, however, in the bladder, estrogen receptor other than ERα seems to be involved.

Material and methods

Mice

C3H/HeJ ovary-intact and OVX mice were purchased from Jackson Laboratories (Bar Harbor, ME). C3H/HeJ mice have served as an established model for UTI pathogenesis [49]. Mice were housed in microisolator cages in United States Department of Agriculture (USDA)-approved facility at the Oklahoma State University Center for Health Sciences. The mice had free access to filtered water and a soy-free diet. All animal experiments and procedures were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee (IACUC).

Drug treatments and experimental UTI induction

PPT and MPP drug (Cayman Chemicals, Ann Arbor, MI) injections were prepared in 1:1 mixture of DMSO and corn oil. Ovary-intact mice (n = 5 per group) were injected with MPP (4 mg/kg body weight) and OVX mice (n = 6 per group) were injected with PPT (10 mg/kg body weight) [50] subcutaneously for 7 consecutive days. Control group mice were injected with vehicle. After drug treatment, experimental UTI was induced in mice transurethrally under anesthesia. Each mouse received 50 μl of 7 × 10^8 cfu/mL of Dr *E. coli* suspension made in phosphate buffered saline (PBS), as previously described [49]. Mice were sacrificed at 2 days or 6 days post-infection (pi). Kidney and bladder tissues were harvested and snap frozen for further analyses.

Determination of bacterial load in urogenital tissues

Kidney and bladder tissues were weighed and homogenized in 0.1% Triton X-100. The tissue homogenates were plated on Luria Bertani (LB) agar plates and incubated overnight at 37°C. Individual bacterial colonies were counted and results were expressed as colony forming units (cfu) per gram of tissue.

Quantitative real time RT-PCR analyses

Total RNA was isolated from kidney tissues with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized from isolated RNA using QuantiNova Reverse Transcription kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative PCR was performed using PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Foster City, CA). The expression levels of target genes, *Cd55* and *Tnfa* were normalized to the endogenous control gene, hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and reported as $2^{-\Delta Ct}$ values. Primer pairs purchased from Integrated DNA Technologies (Coralville, IA) were as follows: *Cd55* (Forward primer-5'GAAAGACTGAGTTTTGCATCCCTCAAAAAAGAG3', Reverse primer-5'CAAAACT GAGCAACTGGAGACCATACTAAATCC3'), *Tnfa* (Forward primer-5'GCCTGTAGCC CACGTCGTAG3', Reverse primer-5'GTCTTTGAGATCCATGCCGTTGGC3') and *Hprt* (Forward primer-5'GCTGACCTGCTGGATTACATTAAAGCACT3', Reverse primer-5'CCCCCGTTGA CTGATCATTACAGTAGC3'). Quantitative RT-PCR was carried out using ABI StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA).

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) bladder and kidney tissues of mice were sectioned (5 mm thick) for immunohistochemical analyses. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol. Heat induced epitope retrieval was performed in 10 mM citrate buffer (pH 6.0) followed by endogenous peroxidase deactivation with Dual Endogenous Enzyme-Blocking Reagent (Dako, Carpinteria, USA). Tissue sections were incubated in 5% horse serum followed by overnight incubation at 4°C with primary antibodies that include rabbit polyclonal anti-mouse CD55 antibody (H-319, Catalog # sc-9156 from Santa Cruz Biotechnology, Santa Cruz, CA) or with goat polyclonal anti-mouse TNFα antibody (Catalog # AF-410-NA from R&D systems, Minneapolis, MN). HRP-conjugated secondary antibodies used include anti-rabbit Ig (Catalog # MP-7401, Vector Laboratories, Burlingame, CA) or HRP-conjugated anti-goat Ig (Catalog # MP-7405, Vector Laboratories, Burlingame, CA). ImmPACT DAB Peroxidase (HRP) Substrate kit (Catalog # SK-4105, Vector Laboratories) was used for antigen detection and nuclei were counter stained using hematoxylin (Vector Laboratories, Burlingame, CA). Stained sections were visualized using an Olympus BX43 microscope and images were taken with an Olympus DP25 camera. Staining intensity at 40 × magnification was quantified using ImageJ IHC profiler software [51].

Statistical analysis

GraphPad Prism version 6 (Graph Pad software Inc, San Deigo, CA) was used for statistical analyses. Group differences for more than two experimental groups were compared using Kruskal-Wallis test (non-parametric ANOVA) with Dunn's post-hoc tests for multiple comparisons. Non-parametric Mann Whitney U-tests were performed for determining differences among two experimental groups, wherever appropriate. Differences at $P < 0.05$ were considered significant.

Results

Modulation of ERα by PPT and MPP differentially regulate UTI disease outcome in bladder versus kidney

We determined the UTI disease outcome in PPT or MPP treated mice by checking the bacterial load in bladder and kidney tissue homogenates.

PPT drug treatment (**Figure 1A**) in mice slightly increased the bacterial colonization in bladder at both 2 days and 6 days pi compared to vehicle treated groups, but not significantly.

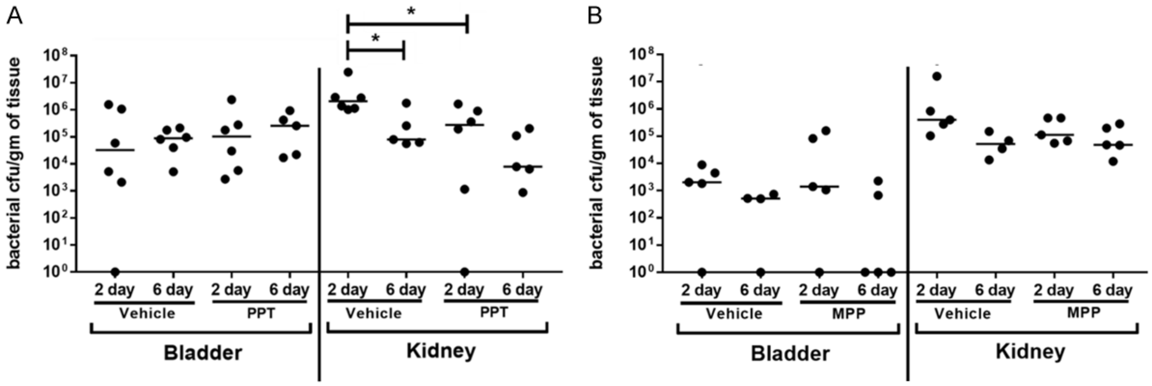


Figure 1. Bacterial load at 2 day versus 6 day post-infection (pi) in bladder and kidney (A) PPT and (B) MPP treated mice (N = 10 to 12 mice per treatment group, each with two time points). Bacterial cfu/gram of tissue were determined in tissue homogenates. PPT significantly reduced *(*P < 0.05)* bacterial load in kidney but not in the bladder. MPP treatment led to efficient clearance of bacterial infection in the bladder, but not in the kidney.

However, in the kidney, PPT treatment resulted in significant *(P < 0.05)* bacterial clearance at 2 days pi compared to the corresponding vehicle treated group with some of these mice showing low or no bacterial counts. The bacterial load in kidney at 6 days pi was found to be the lowest among the groups. Thus, PPT induced enhanced reduction in bacterial load suggesting its protective role in mediating bacterial clearance in the kidney.

MPP treatment (**Figure 1B**) reduced bacterial load in the bladder at 6 days when compared to 2 days pi and vehicle treated groups and three out of the five mice showed complete bacterial clearance. In contrast, we observed no significant change in bacterial load in kidney after MPP treatment at both the time points. Thus, MPP induced enhanced bacterial clearance suggesting its protective role in the bladder.

Modulation of ERα by PPT and MPP differentially regulate CD55 expression in bladder versus kidney

CD55 serves as the host cell receptor for bacterial colonization and its tissue expression is modulated upon infection with Dr *E. coli* [13]. In our study, CD55 protein in the bladder was predominantly expressed in the transitional epithelium, while in the kidney it was mainly expressed in the medullary and cortical tubules. (Representative images shown in **Figure 2B** and **2D**).

PPT treatment did not result in any change in CD55 expression in either bladder or in kidney (**Figure 2A**). However, the overall CD55 expression levels in bladder in both PPT and vehicle treated groups at both time points were more as compared to CD55 expression in the kidney.

MPP treatment considerably reduced CD55 expression in bladder at both time points compared to controls. In contrast, CD55 expression in kidney tissues was reduced only in MPP treated group at 2 days pi as compared to vehicle treated group. However no change in CD55 expression was observed in MPP or vehicle treated groups at 6 days pi.

Cd55 mRNA levels were determined only in kidney tissues of infected mice as bladder tissues were used up for bacterial culture and protein expression studies. No significant differences were observed in *Cd55* mRNA levels in both groups of drug treated mice as compared to controls (**Figure 3A** and **3B**).

Our results highlight the differential regulation of CD55 expression by PPT and MPP in the bladder and kidney during UTI, impacting the infection outcome.

Modulation of ERα by PPT and MPP differentially regulate TNFα expression in bladder versus kidney

TNFα protein expression was predominantly observed in transitional epithelium of the bladder and in medullary and cortical tubules of the kidney (Representative images shown in **Figure 4B, 4D** and **4F**).

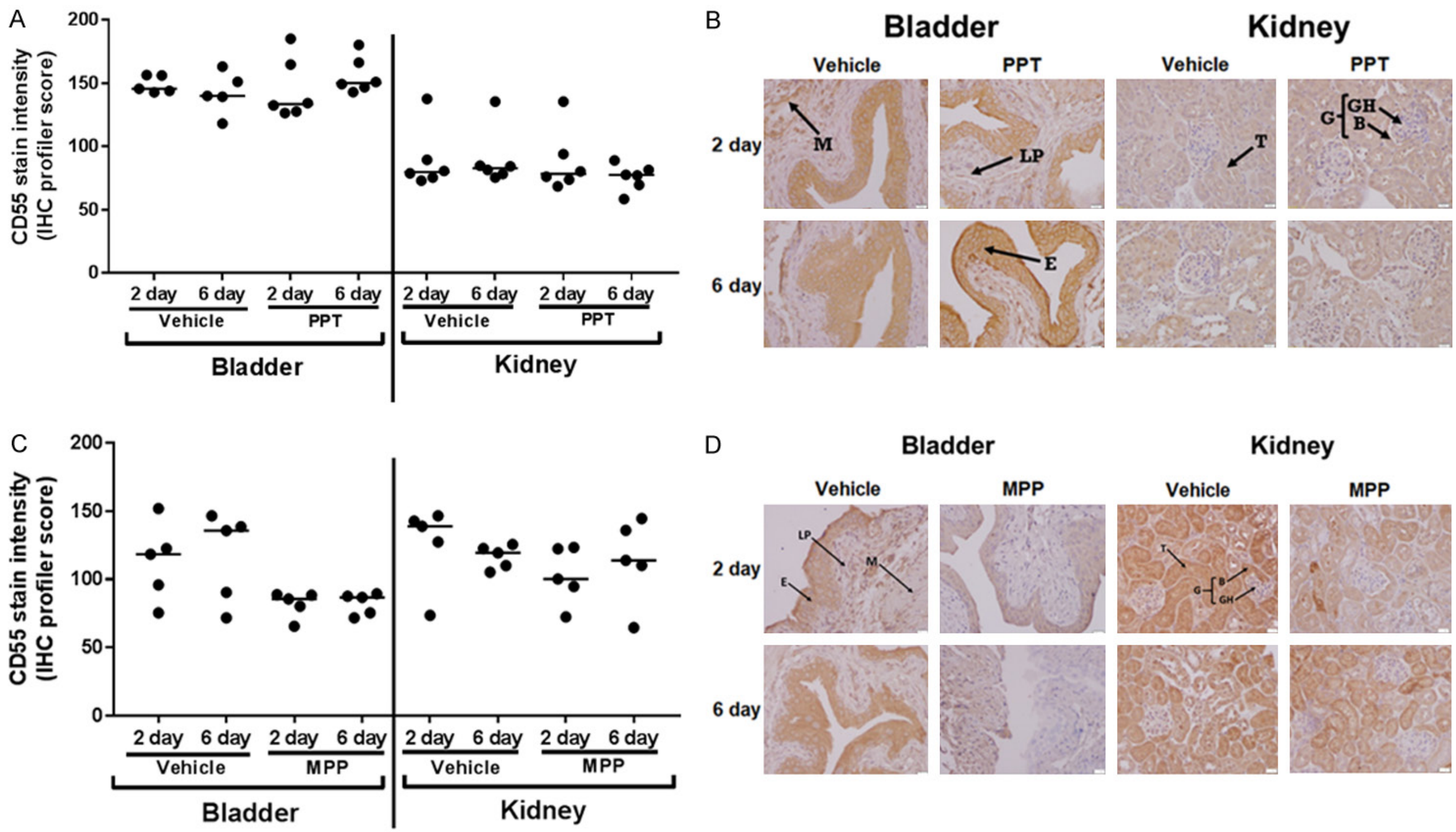


Figure 2. CD55 expression at 2 day versus 6 day pi in bladder and kidney after (A) PPT and (C) MPP treatment in mice (N = 10 to 12 mice per treatment group, each with two time points). Representative images (40X) of bladder and kidney tissue sections showing CD55 protein expression after (B) PPT and (D) MPP treatment (E: Epithelium, LP: Lamina, Propria, M: Muscularis, T: Tubules, G: Glomerulus, GH: Glomerular Head, B: Bowman's Capsule). (A) After PPT treatment, no change in CD55 expression was observed in kidney, while CD55 expression in bladder was considerably high (C) After MPP treatment, CD55 levels were considerably low in bladder but comparatively higher in kidney.

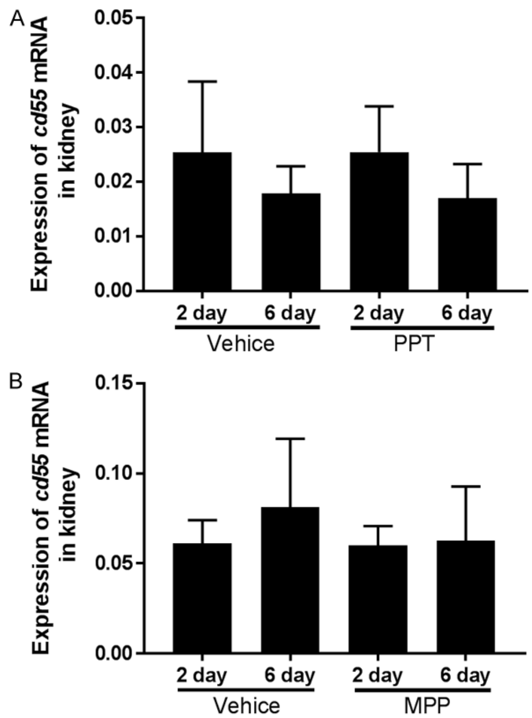


Figure 3. Expression of *cd55* mRNA in kidney after (A) PPT treatment and (B) MPP treatment (N = 10 to 12 mice per treatment group, each with two time points). (A) No changes in CD55 mRNA copy numbers were observed after PPT treatment. (B) However, MPP treatment reduced CD55 mRNA copy numbers at 6 days pi.

PPT treatment significantly *(P < 0.01)* increased TNFα protein expression in bladder at 2 days pi, however there was no change in TNFα expression in kidney at both time points after PPT treatment (**Figure 4A**).

Effects of MPP pre-treatment on TNFα protein expression in bladder and kidney in uninfected mice were determined. MPP treatment in uninfected mice led to significant increase in TNFα expression in the bladder (*P < 0.05*). In infected group, MPP treatment significantly reduced TNFα expression in bladder at 6 days pi compared to vehicle treated group (**Figure 4C** and **4D**). The reduced TNFα expression in infected MPP group corresponded with the bacterial clearance observed in the bladder.

In contrast, MPP treatment in uninfected mice minimally increased TNFα expression in the kidney. In infected group, MPP treatment did not induce any changes in TNFα expression in the kidney at both time points compared to control group, which corresponded with the persistence of infection.

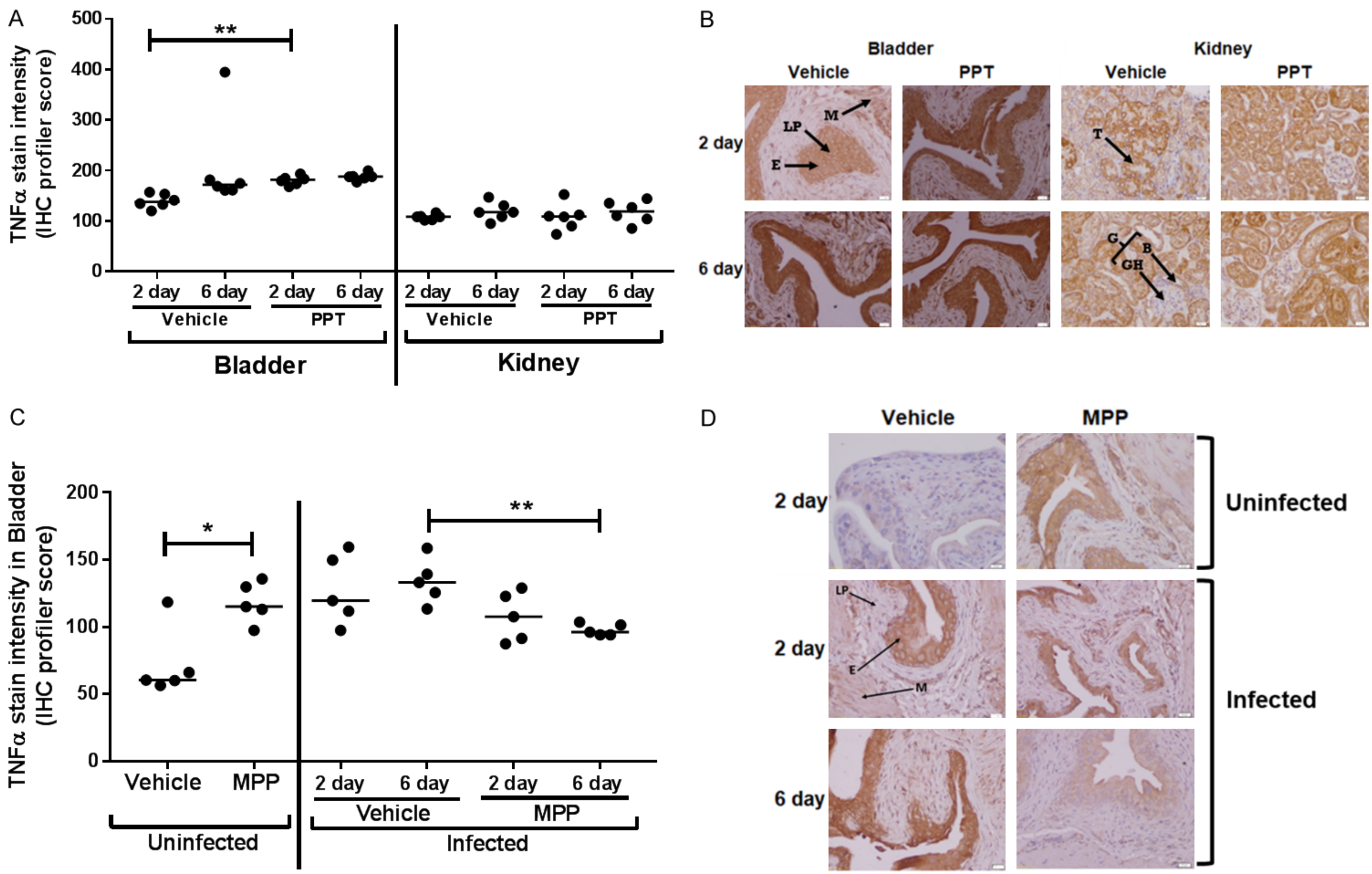
Tnfa mRNA levels were determined only in kidney tissues of infected mice. In PPT treated group, *Tnfa* mRNA levels were comparable to vehicle treated groups at both time points (**Figure 5A**). However, in MPP treated group, *Tnfa* mRNA levels were significantly low *(P < 0.05)* at 2 days pi (**Figure 5B**).

Our results highlight the differential regulation of TNFα expression by PPT and MPP in the bladder and kidney during UTI, impacting the infection outcome.

Discussion

Estrogen mediates its various physiological actions through its receptors, ERα, ERβ or GPR30, which are differentially distributed in various human and mouse tissues [31, 52-56]. The involvement of estrogen and ER subtypes in modulating immune responses has been widely described [27, 28, 30, 57]. The protective action of estrogen in the urogenital tract has been reported [4-8, 17], however, the contributions of ER subtypes in mediating these protective responses have not been sufficiently studied. The aim of this current study was to investigate the involvement of ERα in mediating protection against UTI via modulating the expression of innate immune markers, CD55 and TNFα. We studied the effects of ERα agonist, PPT, and ERα antagonist, MPP, treatment in UTI susceptible C3H/HeJ mice.

PPT treatment in OVX mice reduced bacterial load in kidney at both 2 and 6 days pi, suggesting that bacterial clearance in the kidney is mediated via ERα. In contrast, PPT treatment did not reduce the bacterial load in the bladder, suggesting that ERα may not be involved in bacterial clearance in the bladder. The effects of PPT treatment on CD55 and TNFα expression in the kidney were found to be minimal compared to vehicle treated groups at the selected time points. However, in the bladder, PPT treatment led to elevated levels of CD55 expression at 6 day pi, corresponding to the increased bacterial load observed at this time point. Increased cellular CD55 expression in response to persistent infection with Dr *E. coli* has been previously reported [13]. Also, a significant increase in TNFα expression was



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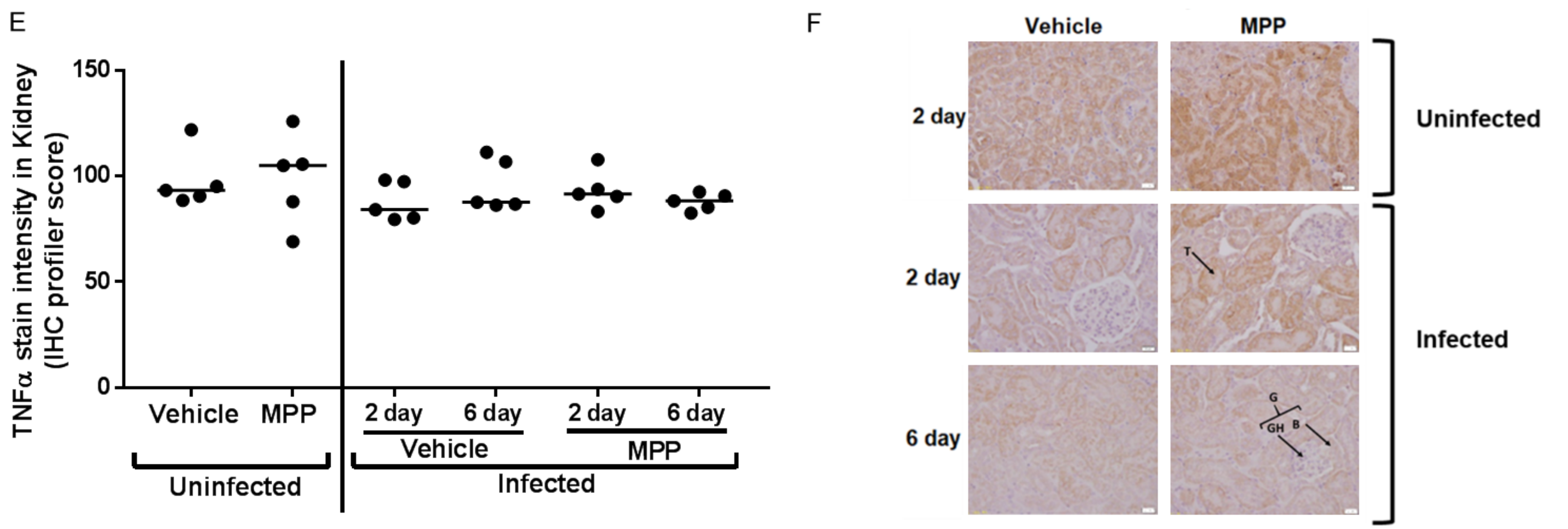

Figure 4. TNFα expression at 2 day versus 6 day pi in bladder and kidney of (A) PPT and (C and E) MPP treated mice (N = 10 to 12 mice per treatment group, each with two time points). Representative images (40 ×) of bladder and kidney tissue sections showing TNFα expression after (B) PPT and (D and F) MPP treatment (E: epithelium, LP: Lamina Propria, M: Muscularis, T: Tubules, G: Glomerulus, GH: Glomerular Head, B: Bowman's capsule). (A) After PPT treatment, TNFα levels were significantly increased in bladder (*$**P < 0.01$*) at 2 days pi but no changes in TNFα levels were observed in the kidney at both time points. (C) In the uninfected state, TNFα levels in bladder were significantly higher *($*P < 0.05$)* after MPP treatment and in infected state TNFα levels significantly reduced *($**P < 0.01$)* as compared to vehicle treated groups. (E) In the kidney, minimal changes in TNFα levels were observed in both uninfected and infected state after MPP treatment.

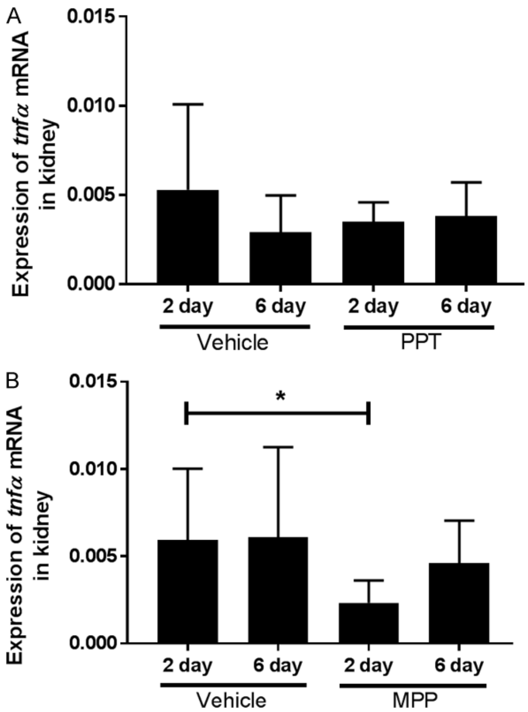

Figure 5. Expression of *Tnfa* mRNA in kidney after (A) PPT treatment and (B) MPP treatment (N = 10 to 12 mice per treatment group, each with two time points). (A) PPT treatment reduced *Tnfa* mRNA copy numbers only at 2 days pi. (B) MPP treatment reduced *Tnfa* mRNA copy numbers at both time points, but significantly at 2 days pi *(*P < 0.05).*

observed in the bladder of PPT treated group due to persistent infection.

MPP treatment resulted in compromised bacterial clearance in the kidney supporting our hypothesis related to involvement of ERα in the kidney. In contrast, MPP treatment led to decreased bacterial load in the bladder, contradicting our hypothesis and suggesting involvement of receptors other than ERα.

Due to persistence of bacterial infection in the kidney of vehicle and MPP treated groups, comparable CD55 and TNFα expression were observed among these groups. In contrast, MPP reduced CD55 and TNFα expression levels in the bladder that corresponded with the observed efficient bacterial clearance in these mice.

We checked the effects of MPP on CD55 and TNFα production in uninfected homeostasis state. Effects of PPT treatment in uninfected mice were not studied as CD55 and TNFα were minimally affected in the kidney during bacterial clearance. Our observations in MPP treated uninfected mice showed a significant upregulation of TNFα expression suggesting that under homeostasis, MPP treatment primes TNFα production in the bladder, thus boosting the pro-inflammatory responses facilitating bacterial clearance. These results are further supported by various reports highlighting the important role of TNFα for mediating immunity in the urinary tract [39, 58-60]. However, the effects of MPP treatment on TNFα expression in the kidneys of uninfected mice were minimal, explaining the observed compromised bacterial clearance in MPP treated infected group. These results further suggest that even during the homeostasis state production of TNFα is differentially regulated in bladder versus kidney in response to the deactivation of ERα by MPP. In light of these findings, boosting TNFα production in the bladder by MPP or similarly acting drugs in combination with antibiotics may serve as a useful strategy for treating recurrent bladder infections.

Differential ER subtypes expression in different tissues including bladder and kidney, has been reported [18-21, 61]. Due to this variable distribution, it is possible that the protective responses against UTI are mediated through different ER subtypes in the bladder versus kidney. ERα and its splice variants are predominantly expressed in kidneys [62-65], supporting our results showing protective effects of ERα in the kidney. In contrast, our results in the bladder suggest involvement of receptors other than ERα. These receptors could be possibly either ERβ (another nuclear ER subtype) or GPER/GPR30 (membrane bound ER), that are also expressed in the bladder [66-68]. Several studies have demonstrated the overexpression of ERβ in the bladder tissues of humans, rats and mice [22, 69-73]. GPER, is also known to cross-talk with the nuclear ER subtypes in order to mediate transcription of target genes [74-77].

To our knowledge, this is the first report indicating differential involvement of ERα in modulating immunity in the bladder and kidney in response to experimental UTI. Further studies are needed to identify the involvement of specific ERs in modulating the immune responses against UTI in the bladder. A comprehensive knowledge of the ER dependent protective signaling mechanisms against UTI may lead us to find novel therapeutic approaches for UTI treatment.

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Disclosure of conflict of interest

None.

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