Research Article

Modulation of persistent bladder pain in mice: The role of macrophage migration inhibitory factor, high mobility group box-1, and downstream signaling pathways

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Abstract

Background: Repeated intravesical activation of protease-activated receptor-4 (PAR4) serves as a model of persistent bladder hyperalgesia (BHA) in mice, which lasts several days after the final stimulus. Spinal macrophage migration inhibitory factor (MIF) and high mobility group box 1 (HMGB1) are critical mediators in the persistence of BHA. **Objective:** We aimed to identify effective systemic treatments for persistent BHA using antagonists or transgenic deletions. **Methods:** Persistent BHA was induced through transurethral instillations of a PAR4-activating peptide (PAR4-AP; 100 μM, 1 h; scrambled peptide, control) under anesthesia, administered on Days 0, 2, and 4. Lower abdominal hypersensitivity was measured on Days 0–4 and 7–9. Systemic injections from Days 2–8 included ISO-1 (a MIF antagonist), ethyl pyruvate (an inhibitor of HMGB1 release), phosphate-buffered saline, or 10% DMSO (vehicle control) in C57BL/6 mice. To examine the role of HMGB1 receptors, Toll-like receptor-4 (TLR4)-null mice or systemic treatment with FPS-ZM1 (receptor for advanced glycation end product [RAGE] antagonist) were used. In addition, TIR-domain-containing adaptor-inducing interferon-β (TRIF)-null mice were tested to assess the involvement of TLR4 signaling pathways. Micturition volume and frequency were assessed on Day 9, and the bladder was histopathologically examined to assess inflammation and edema. **Results:** MIF antagonism significantly reversed persistent BHA, whereas HMGB1 antagonism led to a partial reduction of persistent BHA. TLR4 deficiency or systemic administration of FPS-ZM1 significantly mitigated persistent BHA, while TRIF-deficient mice experienced a faster onset of BHA. Only MIF or HMGB1 inhibition resulted in increased micturition volume. The histopathological examination revealed no changes in inflammation or edema. **Conclusion:** MIF and HMGB1, acting through TLR4 and RAGE, mediated persistent BHA, while TRIF might modulate its onset. Further exploration of downstream TLR4 signaling may uncover novel therapeutic targets for treating persistent bladder pain.

Keywords: Macrophage migration inhibitory factor, High mobility group box-1, Toll-like receptor-4, RAGE, Bladder pain

1. INTRODUCTION

Interstitial cystitis/bladder pain syndrome (IC/BPS) refers to a heterogeneous set of symptoms, including persistent urinary bladder pain and other lower urinary tract symptoms [1]. The etiology of this chronic condition remains poorly understood and can be potentially debilitating. So far, no effective treatment is available [2]. Cystoscopy identifies two common phenotypes: Patients with Hunner lesions and bladder inflammatory changes, and those without Hunner lesions [3,4]. Despite the phenotypic differences, no distinction was reported in terms of bladder pain symptoms between the two groups [5]. The incidence of Hunner lesions reportedly varied from 5% to 57% [4], and most IC/BPS patients do not exhibit bladder inflammation, which may be a causative factor of their bladder pain.

In a previous study, we developed a rodent model of persistent bladder pain that exhibited minimal bladder

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changes, which mimicked the pain component of non-Hunner IC/BPS [6]. In this model, repeated (3 times) activation of bladder protease-activated receptor-4 (PAR4) through intravesical instillation of PAR4-activating peptide (PAR4-AP) induced bladder hyperalgesia (BHA) that persisted for 9 days.

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine found in both immune and non-immune cells [7]. MIF is located in the urothelium as well as in cells within the spinal cord and brain [8,9]. Recent evidence indicated that MIF was involved in pain mediation, with increased spinal MIF levels after induction of experimental cystitis in rodents [10,11].

High mobility group box-1 (HMGB1) is a DNA-binding protein that regulates transcription in most cells. It is also released from cells and serves a wide array of biological functions, depending on its redox state and/or cellular location [12-14]. Extracellularly released HMGB1, termed as damage-associated molecular patterns, or alarmin, activates several receptors, principally Toll-like receptor-4 (TLR4) and receptor for advanced glycation end product (RAGE) [13,15]. While HMGB1 is well-documented in mediating inflammatory processes [14,15], mounting evidence also indicates that HMGB1 plays a crucial role in persistent pain [16,17]. Bladder pain has been reported to be mediated by HMGB1 through RAGE receptors in both inflammation [18] and non-inflammation rodent models [19]. Similarly, TLR4 receptors have been implicated in the mediation of bladder pain in inflammation models [20].

MIF098, a small-molecule inhibitor of MIF, prevents MIF from binding to its cognate receptor, CD74 [21]. Glycyrrhizin works as a direct antagonist of HMGB1 [22]. In our previous study, we demonstrated that systemic treatment with MIF098 partially while glycyrrhizin fully reversed persistent BHA [6]. In addition, we hypothesized that spinal changes were involved in the mediation of persistent BHA and showed that both MIF and HMGB1 levels were elevated in the lumbosacral spinal regions responsible for mediating bladder-related information during persistent BHA [10]. Intrathecal administration of MIF monoclonal antibodies to block MIF or glycyrrhizin to block HMGB1 resulted in remarkable, though temporary (lasting for 4-h) relief of persistent bladder pain [10]. These findings showed that the MIF and HMGB1 worked on the mediation of persistent BHA mainly by acting on the lumbosacral spinal cord.

The aims of the present study were to identify effective systemic treatments for persistent BHA and to further elucidate the downstream mechanisms involved, with a goal to discover potential new therapeutic targets for alleviating persistent BHA.

2. MATERIALS AND METHODS

All animal experiments were approved by the Lexington VA Health Care System Institutional Animal Care and Use Committee (approval ID: VER-19-005-AF) and performed in accordance with the guidelines of the National Institutes of Health, USA.

2.1. Induction of persistent BHA

The protocol for inducing persistent BHA through repeated transurethral instillation of PAR4-AP has been detailed previously [6,23]. Briefly, after induction (3%) and maintenance (1.5%) of isoflurane anesthesia, female mice, aged 12–14 weeks, were instrumented with a transurethral catheter (PE10, Fisher Scientific, USA; 11 mm in length). The bladder was drained by applying gentle pressure to the lower abdominal area. Subsequently, 150 μL of PAR4-AP (AYPGKF-NH_2, Peptides International, USA; 100 μM in sterile phosphate-buffered saline [PBS]; pH 7.4) was slowly instilled into the bladder and left in place for 1 h. Mice were allowed to recover from anesthesia and were then returned to their cages. Intravesical treatments were repeated twice more at 48-h intervals.

Mice that received repeated instillations of a scrambled peptide (YAPGKF-NH2, Peptides International, USA) served as controls (designated "no pain" group) [6,23].

2.2. Evaluation of abdominal mechanical hypersensitivity

The protocol for evaluating lower abdominal mechanical hypersensitivity (interpreted as an index of BHA or bladder pain) to von Frey (VF) monofilaments has been described previously [23]. Briefly, mice were first acclimated to the testing room, chamber, and protocol as follows:

- On Days 1 and 2, mice were placed in the testing room and left undisturbed for 3 h
- On Days 3 and 4, mice were placed in the testing chamber and left undisturbed for 2 h
- On Day 5, mice were placed in the testing chamber, and VF monofilaments were applied to the lower abdominal area.
- On Day 7, baseline VF testing (Day 0) was conducted before the initiation of transurethral instillations.

The response to stimulation of the lower abdominal region using VF monofilaments (at 0.008, 0.02, 0.07, 0.16, 0.4, 1.0, 2.0, and 6.0 g) was used to calculate the 50% mechanical threshold [24]. A positive response was defined by one of three behaviors: (i) licking the abdomen; (ii) flinching/jumping; or (iii) abdomen withdrawal. If a positive response to a stimulus occurred, the next smaller VF filament was applied. Otherwise, the next larger filament was applied. Thresholds (50%) were measured at baseline (Day 0; before any treatment) and on Days 2, 3, 4, 7, 8, and 9.

2.3. Treatments for alleviating persistent BHA

MIF binds multiple receptors aside from its cognate receptor, CD74 [25]. While we previously demonstrated partial reversal of persistent bladder pain using a MIF-CD74 antagonist, we aimed to examine the effect of systemic treatment with a direct MIF inhibitor, ISO-1 [26], to block all receptor-mediated effects of MIF. We also sought to determine whether blocking HMGB1 release through systemic treatment with ethyl pyruvate (EP) [22] would effectively reverse persistent BHA. In addition, we examined the contribution of HMGB1-mediated signaling through TLR4 using TLR4-deficient mice. HMGB1-mediated signaling through RAGE was examined by systemic treatment with FPS-ZM1. Further downstream from TLR4, we examined the contribution of TIR-domain-containing adaptor-inducing interferon-β (TRIF), using TRIF-deficient mice, since TRIF has been implicated in modulating neuropathic pain [27].

Systemic treatments (intraperitoneal [i.p.] injections) or knockout (KO) strains were used to test their ability to alleviate persistent BHA. C57BL/6 mice (aged 12–14 weeks of age, wild type strains [WT], The Jackson Lab, USA) received systemic treatment with a MIF antagonist (ISO-1, 20 mg/kg) or solvent (vehicle control) from Days 2 to 8 of the persistent BHA protocol (Figure 1). In addition, mice were treated with an HMGB1 release inhibitor (EP; 200 mg/kg) or its solvent (vehicle control) from Days 2 to 8. WT mice were treated with the RAGE antagonist (FPS-ZM1, 10 mg/kg) or its solvent (vehicle control) during the same period.

Tlr4-KO (B6[Cg]-TLR4$^{tm1.2Karp}$/J, aged 12–14 weeks, The Jackson Lab) and *Trif*-KO (Trif^{Lps2}; 12–14 weeks of age; The Jackson Lab, USA) mice received intravesical PAR4-AP instillations to induce persistent BHA, while intravesical scrambled peptide was used in the "no pain" control group. These groups of mice were treated with systemic PBS injections as controls for i.p. injections.

2.4. Determination of awake micturition parameters

On Day 9, following the final abdominal mechanical threshold testing, micturition parameters (micturition volume and frequency) were measured in awake mice using the voided stain on paper (VSOP) method [23,28]. Individual mice were placed in plastic enclosures that allowed free movement and access to water. Micturitions were collected on filter paper placed beneath the enclosure during a 3-h observation period. Micturition volume was calculated by measuring the spot size on the filter paper and applying linear regression based on a set of known volumes. Micturition frequency was determined by counting the number of micturitions over the 3-h observation period.

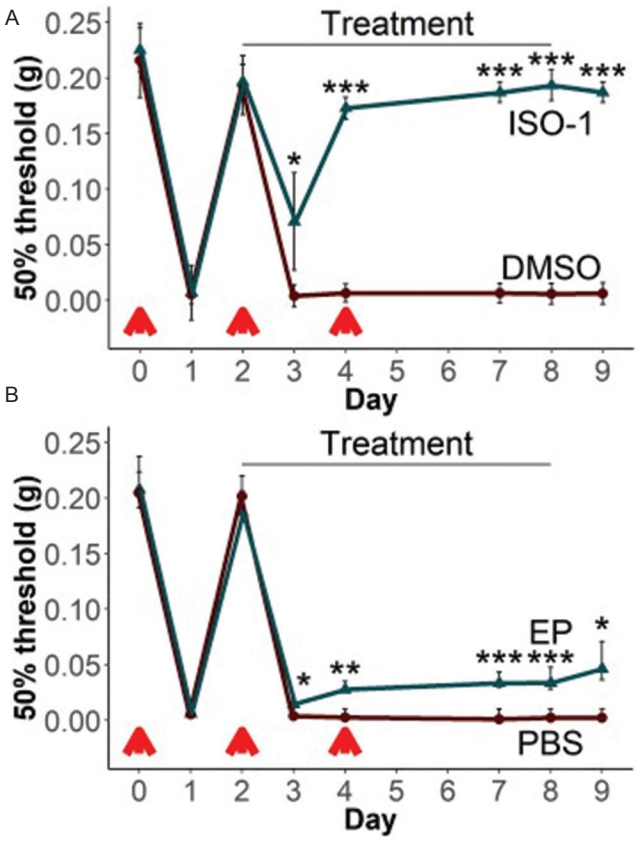

Figure 1. Effect of MIF and HMGB1 antagonism on persistent bladder hyperalgesia (BHA). The Y-axis represents the mean and standard deviation of the 50% threshold (in grams) for lower abdominal stimulation using VF monofilaments, while the X-axis represents time in days. Red arrows indicate the days of intravesical PAR4-AP instillations. Systemic treatments were administered from Days 2 through 8. Differences in threshold values due to treatment were analyzed using a two-way repeated measures analysis of variance, followed by Tukey's *post hoc* tests. Repeated (×3) instillations of PAR4-AP resulted in a marked and significant decrease in the threshold (an index of bladder pain), which temporarily recovered on Day 2 but decreased again following subsequent instillations, remaining decreased through Day 9. (A) Mice treated with 10% DMSO (red line) as a vehicle control showed no change in threshold. However, treatment with the MIF antagonist (ISO-1; 20 mg/kg; green line) resulted in a significant increase in threshold compared to the control group (DMSO). (B) Mice treated with PBS (solvent control) exhibited persistent BHA that persisted for nine days (red line), while those treated with the HMBG1 antagonist (ethyl pyruvate [EP]; 200 mg/kg; green line) showed a modest but statistically significant increase in threshold compared to the control group. HMGB1: High mobility group box 1; MIF: Migration inhibitory factor; PAR4: Protease-activated receptor-4.
Notes: *p<0.05, **p<0.01, ***p<0.001.

2.5. Bladder histology scoring

Following the measurement of awake micturition parameters (VSOP, as described above), mice were anesthetized with 3–4% isoflurane, and their bladders were rapidly removed. Bladder segments were then placed in 4% buffered formaldehyde (#SF100-4, Fisher Scientific, USA) for histological analysis. Paraffin-embedded bladder sections

(5 μm) were processed for routine hematoxylin and eosin (H&E) staining. The stained sections were evaluated by two independent observers blinded to experimental grouping and scored separately for edema and inflammation on the following scale, as previously described [6,23]:

- 0: No edema or infiltrating cells
- 1: Mild submucosal edema and few inflammatory cells
- 2: Moderate edema and a moderate number of inflammatory cells
- 3: Frank edema, vascular congestion, and many inflammatory cells

2.6. Statistical analysis

Values were reported as mean ± *SD* or median (IQR) for categorical variables (*e.g.*, edema, inflammation scores). All statistical tests were performed using R [29].

Differences in VF thresholds due to treatment were analyzed using repeated-measures two-way analysis of variance (ANOVA), with treatment type and day as independent variables and VF threshold as the dependent variable. If the ANOVA was significant ($P < 0.05$), it was followed by Tukey's *post hoc* tests. For transgenic strains, the analyses involved types of intravesical instillations and day of treatment as independent variables; with VF threshold as the dependent variable (All received i.p. PBS injections). Differences between treatment groups from Day 2 to 9 were reported.

Micturition parameters were analyzed using one-way ANOVA, followed by *post hoc* Dunnett's tests if the ANOVA was significant. Histological scores were analyzed using Fischer's exact test, followed by pairwise comparisons if the difference was significant.

3. RESULTS

3.1. Effect of systemic treatment with MIF antagonist on persistent BHA

Figure 1A illustrates the effect of systemic injections of the MIF antagonist (ISO-1; 20 mg/kg; green line) or it's solvent (10% DMSO; red line) on persistent BHA. Repeated intravesical instillations of PAR4-AP are indicated by red arrows. Both groups experienced a significant drop in threshold (indicative of BHA) 1 day after the first instillation (Day 1) compared to baseline (Day 0). While the threshold in both groups recovered on Day 2, it significantly decreased again on Day 3 and remained decreased until Day 9 in the group treated with systemic DMSO (solvent control). In contrast, systemic treatment with the MIF antagonist ISO-1 (20 mg/kg, i.p. daily, Days 2–8) led to a significant increase in threshold (indicative of bladder pain relief), beginning on Day 3 and continuing through Day 9. By the end of the treatment, the threshold in the ISO-1-treated groups had essentially returned to baseline (Figure 1A).

3.2. Effect of systemic treatment with HMGB1 antagonist on persistent BHA

EP is an HMGB1 antagonist that inhibits HMGB1 release [30,31]. We assessed the effect of systemic EP injections on persistent BHA. Figure 1B shows the effect of i.p. injections of EP (200 mg/kg) or its solvent (PBS) on the 50% threshold. Treatments were administered from Days 2 through 8. Mice treated with PBS (vehicle control; i.p.) exhibited the typical pattern of a reduced 50% threshold, characteristic of persistent BHA (Figure 1B; red line). In contrast, EP treatment resulted in a modest but statistically significant increase in the 50% threshold (Figure 1B; green line), indicative of partial bladder pain relief, on Days 3 through 9 compared to the PBS-treated group (Figure 1B; red line).

3.3. Effect of TLR4 deficiency on persistent BHA

To assess the role of TLR4 receptors in persistent BHA, we examined whether repeated intravesical PAR4 activation would result in persistent BHA in TLR4-deficient mice. A scrambled peptide was used for the "no pain" control group. Both groups were treated with i.p. injections of PBS from Days 2 to 8 as controls for treatment parameters.

Tlr4-KO mice treated with the scrambled peptide showed no change in the 50% threshold for lower abdominal stimulation with VF monofilaments, as expected for the "no pain" group (Figure 2A; red line). However, *Tlr4*-KO mice that received repeated transurethral PAR4-AP instillations showed a decreased threshold consistent with the development of persistent BHA (Figure 2; green line). A significant decrease in threshold was observed 1 day after the first transurethral administration of PAR4-AP, followed by recovery on Day 2. After the second administration of PAR4-AP, the threshold decreased again and remained reduced through Day 9. This pattern mirrored the response observed in WT mice (Figure 1A and 1B), although the magnitude of the decrease was approximately 50% less in *Tlr4*-KO mice. Specifically, the sustained reduction in threshold was about 0.1 g in *Tlr4*-KO mice compared to close to 0.00 g in WT mice (Figure 1). These results suggest that TLR4 deficiency offers partial protection against persistent BHA in mice.

3.4. Effect of systemic treatment with RAGE antagonist on persistent BHA

Since HMGB1 also activates RAGE receptors [13], we looked at the effect of treating WT mice with a RAGE antagonist [32] on persistent BHA. Figure 2B shows the effect of systemic treatment with FPS-ZM1 (green line) or

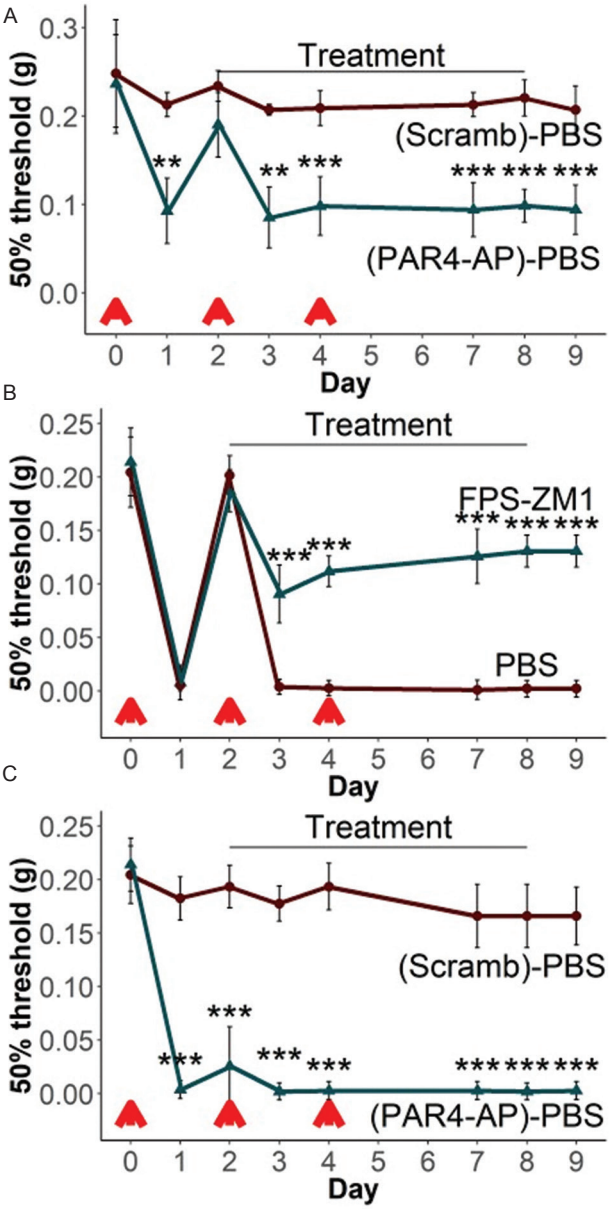

Figure 2. Effect of TLR4 deficiency, systemic RAGE antagonism, and TRIF deficiency on persistent bladder hyperalgesia (BHA). (A) TLR4-deficient mice were treated systemically with PBS as a treatment control. These mice showed no changes in threshold following repeated (×3) intravesical instillations of a scrambled peptide ("no pain" control; red line). However, a significant and persistent decrease in threshold was observed following repeated instillations of PAR4-AP. The effect was reduced compared to wild-type mice. (B) Both groups were treated with PBS. Wild-type mice that received repeated instillations of PAR4-AP exhibited the typical pattern of persistent BHA (red line). In contrast, treatment with the RAGE antagonist (FPS-ZM1; 10 mg/kg; i.p.; green line) resulted in a significant increase in threshold compared to the control group. (C) TRIF-deficient mice showed no changes in threshold following repeated (×3) instillations of scrambled peptide ("no pain" control group; red line). However, they exhibited a marked and persistent decrease in threshold upon repeated instillations of PAR4-AP, indicating persistent BHA. TLR4: Toll-like receptor-4; RAGE: Receptor for advanced glycation end product; TRIF: TIR-domain-containing adaptor-inducing interferon-β; PBS: Phosphate-buffered saline.
Notes: **p<0.01, ***p<0.001.

PBS (vehicle control, red line) on persistent BHA induced by PAR4-AP. As expected, systemic PBS treatment did not alter the persistent BHA induced by repeated transurethral administration of PAR4-AP (Figure 2B; red line). However, systemic treatment with FPS-ZM1 (10 mg/kg, i.p.) resulted in a significant partial reduction of persistent BHA starting on Day 3 and continuing through Day 9 (Figure 2B; green line), compared to the PBS-treated control group.

3.5. Effect of TRIF deficiency on persistent BHA

Since TLR4 receptors activate signaling through both TRIF- and MyD88-dependent pathways [33], we tested the contribution of TRIF-dependent signaling to persistent BHA using TRIF-deficient mice. These mice received either repeated transurethral infusions of PAR4-AP to induce persistent BHA or scrambled peptide transurethral infusions as a "no pain" control. Both groups were treated with systemic PBS injections from Day 2 through Day 8 to control for systemic administration parameters.

Figure 2C shows the 50% threshold responses to lower abdominal stimulation with monofilaments in *Trif*-KO mice that received either transurethral scrambled peptide (red line) or transurethral PAR4-AP peptide. As expected, repeated transurethral administration of the scrambled peptide did not affect the 50% threshold, which was consistent with the "no pain" group. In contrast, treatment with PAR4-AP resulted in a significant reduction in the 50% threshold, indicative of unalleviated persistent BHA. Compared to the repeated scrambled peptide group (Figure 2C, red line), PAR4-AP treatment caused a marked and significant decrease in the threshold from Days 1 to 9 (Figure 2C; green line). Interestingly, the recovery in the 50% threshold observed on Day 2 in WT mice (Figures 1 and 2B) and even in TLR4-deficient mice (Figure 2A) was absent in *Trif*-KO mice.

3.6. Effects of treatments on awake micturition parameters

We investigated the effect of the various treatments on awake micturition volume and frequency, using the VSOP method [28] with a 3-h observation period. Table 1 lists the mean ± *SD* micturition volume and frequency for all five WT groups in this study. Repeated transurethral treatments are presented in parentheses, followed by systemic treatments. All comparisons were made against the group that received repeated transurethral PAR4-AP instillations and systemic PBS treatment.

Systemic MIF antagonism with ISO-1 or the HMGB1 antagonist, EP, resulted in a significant increase in micturition volume compared to the PBS-treated group (Table 1). Mice treated with ISO-1 had a mean micturition volume

of 301 ± 77 µL, while those treated with EP had a mean micturition volume of 230 ± 78 µL. Both volumes were significantly higher than those of the PBS-treated mice (139 ± 36.9 µL, Table 1). No other treatments significantly affected micturition volume, and none of the treatments impacted micturition frequency.

Furthermore, we examined micturition parameters in two transgenic strains, i.e., TLR4-deficient and TRIF-deficient mice and compared the effect of repeated transurethral PAR4-AP to that of scrambled peptide instillations. In both *Tlr4*-KO and *Trif*-KO mice (Table 1), repeated instillations with PAR4-AP did not cause significant changes in micturition volume or frequency when compared to their respective scrambled-peptide control groups (Table 1).

3.7. Effects on bladder histological scores

Bladder sections stained with H&E from all groups of mice were scored for histological findings of inflammation and edema. The scores are presented as median (IQR) in Table 2.

Table 1. Awake micturition parameters for all groups

Strain	Treatment	Micturition Volume (µL) (Mean±*SD*)	Frequency (Micturition/3 h) (Mean±*SD*)	*n*
C57BL/6	(PAR4-AP)-PBS	139±36.9	3.2±0.8	6
	(PAR4-AP)-DMSO	159±42.8	3.0±0.63	6
	(PAR4-AP)-ISO-1	301±77.0***	1.3±0.52	6
	(PAR4-AP)-EP200	230±78.0*	3.3±1.21	6
	(PAR4-AP)-FPS-ZM1	208±31.3	2.2±1.33	6
Tlr4-KO	(Scramb)-PBS	232±74.1	2.0±0.32	5
	(PAR4-AP)-PBS	245±161.0	1.7±0.59	8
Trif-KO	(Scramb)-PBS	237±133.0	2.0±1.1	6
	(PAR4-AP)-PBS	178±46.0	3.5±1.52	6

Notes: *p<0.05, **p<0.01, ***p<0.001. PAR4: Protease-activated receptor-4; PBS: Phosphate-buffered saline.

Table 2. Histological scores for bladder inflammation and edema for all groups

Strain	Treatment	Inflammation (Median [IQR])	Edema (Median [IQR])	*n*
C57BL/6	(PAR4-AP)-PBS	0 (0)	0 (0.75)	6
	(PAR4-AP)-DMSO	0 (0)	0.5 (1.0)	6
	(PAR4-AP)-ISO-1	0 (0)	0 (0.75)	6
	(PAR4-AP)-EP200	0 (0)	0 (0)	6
	(PAR4-AP)-FPS-ZM1	0 (0)	0 (0.19)	6
Trl4-KO	(Scramb)-PBS	1 (2.0)	2.0 (2.0)	5
	(PAR4-AP)-PBS	0.5 (1.0)	1.5 (1.25)	8
Trif-KO	(Scramb)-PBS	0 (0)	0 (0)	6
	(PAR4-AP)-PBS	0 (0)	0 (0.75)	6

IQR: Interquartile range; PAR4: Protease-activated receptor-4; PBS: Phosphate-buffered saline.

Table 2 lists the scores of all WT groups in this study. Comparisons were made between the WT group that received repeated transurethral PAR-AP and systemic PBS (untreated pain group) treatments. There were no significant differences in inflammation or edema scores among the WT groups, with both indices remaining negligible in all groups. Figure 3 provides representative images of bladder sections from the groups listed in Table 2.

Similarly, we examined H&E-stained sections from the bladders of transgenic mice for histological signs of inflammation and edema. Table 2 presents the median and IQR scores for the *Tlr4*-KO groups. No significant differences were

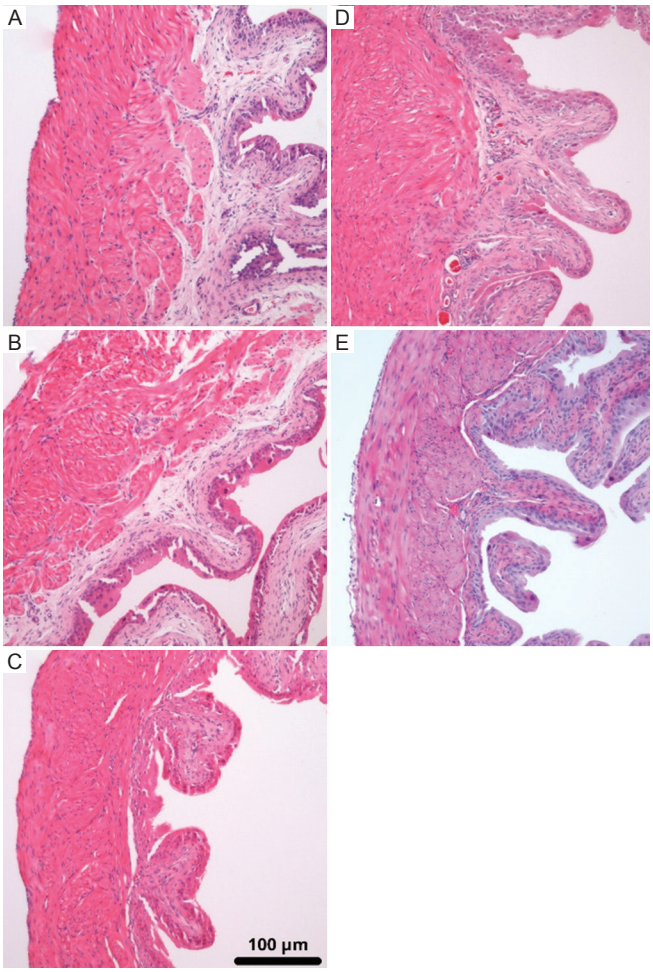

Figure 3. Histological assessment of bladder inflammation and edema. Bladders from all groups were collected on Day 9 (the endpoint of the experiment), processed for hematoxylin and eosin staining, and scored for inflammation and edema as described in the text and listed in Table 2. Representative section images are presented for all wild-type groups receiving different treatments. All groups received repeated (×3) intravesical PAR4-AP. (A) Mice treated systemically with PBS. (B) Mice treated systemically with DMSO. (C) Mice treated with the MIF antagonist ISO-1. (D) Mice treated with the HMGB1 antagonist ethyl pyruvate. (E) Mice treated with the RAGE antagonist FPS-ZM1. Scale bar: 100 µm; magnification: ×100. HMGB1: High mobility group box 1; MIF: Migration inhibitory factor; PAR4: Protease-activated receptor-4; PBS: Phosphate-buffered saline.

observed between mice treated with repeated transurethral PAR4-AP (persistent BHA group) and those treated with scrambled peptide ("no pain" group) in terms of inflammation or edema. While the scores remained low in both indices, a higher degree of edema (not tested statistically) was noted in these groups compared to WT mice (Table 2). Figure 4A and C show representative images of bladder sections from mice treated with repeated transurethral scrambled peptide or PAR4-AP, respectively.

Finally, histological examination of bladder sections from TRIF-deficient mice treated with either repeated transurethral scrambled peptide ("no pain" group) or PAR-AP ("persistent pain" group) revealed no significant differences in the scores for inflammation or edema (Table 2), with both indices remaining negligible. Representative images of bladder sections from these groups are presented in Figure 4B and D.

4. DISCUSSION

Pain is a cardinal symptom of inflammation [34], and bladder pain is often studied by using models characterized by considerable bladder damage and injury, such as the cyclophosphamide model [35,36]. Nonetheless,

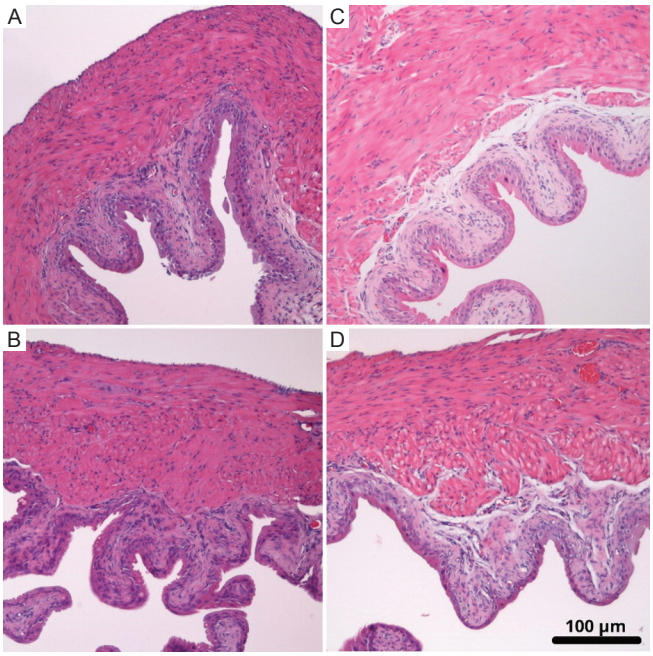

Figure 4. Histological examination of bladders from TLR4- and TRIF-deficient strains. Representative hematoxylin and eosin-stained sections are presented from each group. All groups received systemic PBS as a treatment control (A) TLR4-deficient mice receiving repeated intravesical scrambled peptide. (B) TLR4-deficient mice receiving repeated intravesical PAR4-AP. (C) TRIF-deficient mice receiving repeated intravesical scrambled peptide. (D) TRIF-deficient mice receiving repeated intravesical PAR4-AP. Histological scores for inflammation and edema are listed in Table 2. Scale bar: 100 µm; magnification: ×100. PBS: Phosphate-buffered saline; TLR4: Toll-like receptor-4; TRIF: TIR-domain-containing adaptor-inducing interferon-β.

the persistent BHA model used in this study induced pain with minimal or no histological signs of bladder inflammation (Table 2; Figures 3 and 4), corroborating our earlier reports [6,10]. One limitation of this model is the reliance on histological assessment alone to determine bladder inflammation. Future studies incorporating immunohistochemical study of bladder inflammation markers (*e.g.*, CD45) will allow for the verification of histological scoring and provide a more definitive assessment of bladder inflammation levels in this model. Our current findings, along with those from other pain models [37,38], demonstrated that it is possible to dissociate pain from inflammatory changes, even in the bladder [19,39]. This distinction is crucial for understanding chronic pain that occurs in the absence of overt inflammation, as is the case for the majority of non-Hunner IC/BPS patients [5].

The persistent BHA rodent model used in this study, although initiated by repeated activation of peripheral bladder receptors, was associated with proteomic changes in the lumbosacral spinal cord, which contributed to the development of bladder pain [40]. Moreover, spinal MIF antagonism has been shown to temporarily reverse both persistent BHA and the associated proteomic changes in the lumbosacral spinal cord [23]. These findings suggest that MIF plays a vital role in mediating persistent bladder pain, as the reversal of spinal MIF-modulated protein changes is associated with pain relief in this model.

We evaluated the effect of blocking MIF, HMGB1, and downstream signaling pathways on the reversal of persistent bladder pain in mice. Our current results showed that systemic treatment with the MIF inhibitor ISO-1 completely reversed established bladder pain (Figure 1A). Increasing evidence supports the role of MIF in the modulation of neuropathic pain [41]. For instance, spinal administration of an MIF antagonist (ISO-1) has been shown to prevent and reverse inflammatory pain in rats [42]. Similarly, ibudilast, a PDE4 inhibitor initially developed for asthma treatment that also acts as an MIF [43-45] and TLR4 inhibitors [46], have been found to be effective in decreasing persistent neuropathic pain by targeting the spinal cord [47]. These findings highlight the potential of MIF and MIF-mediated signaling as promising avenues for further research and therapeutic development aimed at ameliorating persistent bladder pain. Given that ibudilast, which blocks both MIF and TLR4 receptors, is currently in phase 2 clinical trials for progressive multiple sclerosis [48], future studies may explore its potential, or that of similar MIF inhibitors, to treat IC/BPS-associated bladder pain.

We also examined the effect of HMGB1 inhibition on the reversal of established bladder pain. Treatment with EP, an HMGB1 antagonist that inhibits HMGB1 release and also

functions as an antioxidant [22], partially reduces bladder pain (Figure 1B). In previous studies, we demonstrated that systemic treatment with a different HMGB1 antagonist (glycyrrhizin) fully reversed established bladder pain in this model [6]. The modest, yet significant, effect observed with EP in the current study suggests that differences in treatment dose and/or bioavailability of EP may account for the discrepancy in the efficacy between the two HMGB1 antagonists.

HMGB1 is recognized as a potential mediator of persistent pain, acting at both peripheral and central nervous system sites [16,17]. Tanaka *et al*. [36] showed that HMGB1 antagonism prevented bladder pain, but not inflammation, in a cyclophosphamide-induced model of chemical cystitis in rodents. Our current results with EP, in combination with previous findings using a different HMGB1 antagonist (glycyrrhizin) [6], support the notion that HMGB1 plays a role in mediating bladder pain.

Interestingly, both MIF antagonism with ISO-1 and HMGB1 antagonism with EP resulted in increased micturition volume in this study (Table 1). In earlier research, we reported that MIF-deficient mice exhibited a significantly larger (virtually double) micturition volume and a concomitantly lower micturition frequency compared to their WT counterparts [10]. When combining these previous findings with our current result that systemic treatment with the MIF-inhibitor (ISO-1) increased micturition volume in WT mice, an intriguing possibility emerges: MIF may mediate micturition sensation. Thus, the role of MIF in overactive bladder conditions warrants further investigation.

We also examined the role of TLR4 and RAGE, both being receptors for HMGB1, in mediating persistent BHA. Our results showed that TLR4 deficiency blunted the BHA response by approximately 50% compared to WT mice (Figure 2A). These findings are consistent with previous reports that TLR4 deficiency resulted in a 50% reversal of pain responses in a neuropathic pain model of male mice [27]. However, in our study, we exclusively used female mice, highlighting the need to carefully consider sex and model differences when assessing TLR4 signaling in pain mediation. TLR4 has been shown to mediate pain independent of inflammation in a bacterial cystitis model [49], and TLR4 antagonism with TAK-242 or *Tlr4*-KO mice reduced bladder pain in an autoimmune cystitis model [20]. In IC/BPS patients, TLR4-mediated responses in peripheral cells have been found to be associated with increased reports of extra-pelvic pain [50]. Together, our findings and previous studies suggest that TLR4 receptors play an important role in the mediation of bladder pain.

In our model, treatment with a RAGE inhibitor (FPS-ZM1) significantly reversed persistent BHA (Figure 2B). Other investigators have reported that RAGE inhibitors, rather than TLR4 inhibitors, prevented bladder pain following

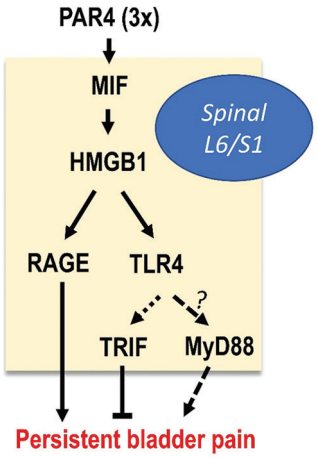

Figure 5. Signaling pathway involved in the development of persistent bladder hyperalgesia. Repeated activation (3×) of bladder PAR4 receptors establishes a spinal (L6/S1) mechanism involving MIF and HMGB1 that maintains persistent bladder pain. MIF acts upstream of HMGB1. Blocking either MIF or HMGB1 reduces persistent bladder pain. Both TLR4 and RAGE (both HMGB1 receptors) mediate persistent bladder pain. TRIF may modulate the onset of persistent bladder pain, though the role of MyD88 remains to be determined. HMGB1: High mobility group box 1; MIF: Migration inhibitory factor; PAR4: Protease-activated receptor-4; RAGE: Receptor for advanced glycation end product; TLR4: Toll-like receptor-4.

cyclophosphamide-induce cystitis [18,36], as well as after intravesical administration of substance P [19], a milder pain model that induces bladder pain 24 h post-infusion with minimal bladder inflammation, similar to the PAR4 model used in our study. These findings underscore the evidence that both TLR4 and RAGE contribute to bladder pain, and our results confirm their role in mediating persistent bladder pain. Further research is needed to explore downstream signaling pathways of these receptors, as well as potential model- and sex-specific differences.

Finally, in an attempt to further investigate TLR4-related signaling pathways associated with the mediation of persistent BHA, we examined TRIF-deficient mice. Contrary to expectations, TRIF deficiency did not alleviate persistent BHA; in fact, these mice developed persistent BHA more rapidly (Figure 2C). Unlike WT or TLR4-deficient mice, TRIF-deficient mice did not exhibit the rebound in the 50% threshold seen on Day 2, suggesting that TRIF deficiency accelerates the onset of persistent BHA. Therefore, it appears that TRIF modulates the timing of persistent bladder pain development in mice. The precise mechanism underlying this effect remains unclear and warrants further investigation. In male mice, TRIF deficiency has been linked to increased neuropathic pain, mediated at the spinal cord level through downstream interferon β signaling [27]. In addition, MyD88 has been implicated in neuropathic pain [27], though its contribution to persistent bladder pain was not explored in the current study. Future studies should focus on other HMGB1-/TLR4-mediated signaling pathways, such as

MyD88, the balance between TRIF and MyD88 signaling, and HMG1-/RAGE-mediated signaling in persistent bladder pain.

5. CONCLUSION

Repeated (3 times) activation of PAR4 receptors in the bladder appears to establish a central (most likely spinal) mechanism that mediates persistent bladder pain. Our findings indicated that persistent bladder pain was mediated by spinal MIF and HMGB1 (Figure 5). Systemic treatments with MIF and/or HMGB1 antagonists effectively reversed persistent BHA in mice, with MIF antagonism showing greater efficacy than HMGB1 antagonism. Persistent bladder pain is mediated by both HMGB1 receptors, TLR4 and RAGE. TRIF signaling does not alleviate persistent bladder pain and may in fact modulate its onset. Targeting MIF and its downstream signaling pathways may represent a promising therapeutic approach for mitigating persistent bladder pain in conditions such as IC/BPS.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

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Writing – original draft: All authors
Writing – review & editing: All authors

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments were approved by the Lexington VA Health Care System Institutional Animal Care and Use Committee (approval ID: VER-19-005-AF) and performed in accordance with the guidelines of the National Institutes of Health.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA

The raw data supporting the conclusions of this article will be made available by the authors on request.

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