Effect of Herpes Simplex Virus Vector-mediated Interleukin-4 Gene Therapy on Bladder Overactivity and Nociception

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ABSTRACT (199 words)

We investigated the effects of replication-defective herpes simplex virus (HSV) vector expression of interleukin-4 (IL-4) on bladder overactivity and nociception. HSV vector expressing murine interleukin-4 (S4IL4) or the control vector expressing β-galactosidase (SHZ) were injected to the rat bladder wall. One week after viral injection, in cystometry performed under urethane anesthesia, the S4IL4 treated group did not show the Intercontraction intervals (ICI) reduction during intravesical administration of 10 nM resiniferatoxin (RTx). Two weeks after viral injection, behavioral studies were performed on vector-injected animals in an awake-state. Freezing behavior induced by 3 μM RTx administered for 1 minute into the bladder was significantly suppressed in the S4IL4 group compared to the SHZ group. Murine IL-4 levels examined by ELISA were significantly increased in bladder and bladder afferent dorsal root ganglia (DRGs) at 2 weeks after viral injection. The expression of IL-1β and IL-2 and bladder inflammatory responses were significantly suppressed in the RTx irritated bladder of S4IL4-injected rats. These results indicate that HSV vector-mediated interleukin-4 expression in the bladder and bladder afferent pathways reduces the inflammatory response, bladder overactivity and nociceptive behavior induced by bladder irritation in the rat model. Therefore IL-4 gene therapy could be a new strategy

for treating urinary frequency and/or bladder pain.

Key Words: gene therapy; urinary bladder; herpes simplex virus; interleukin-4

INTRODUCTION

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a serious disease characterized by increased bladder pain and urinary frequency that is often difficult to treat due to the variation and severity in patient symptoms. Although the etiology of IC/PBS is not fully understood, bladder inflammation associated with production of inflammatory cytokines has been proposed as a potential cause of pathogenesis of the disease.[1,2] In previous studies, cytokines and chemokines such as interleuken-2 (IL-2), IL-6, IL-8 and tumor necrosis factor-alpha (TNFα) were significantly increased in IC/PBS patients' bladder tissue and urine than in controls, suggesting that these cytokines might represent specific markers of IC/PBS.[2-4] In contrast, IL-4 is a prototypical anti-inflammatory cytokine, which is known to inhibit secretion of inflammatory cytokines like IL-1β, TNFα, and IL-6.[5-7] IC/PBS patients have displayed lower levels of IL-4[8] that increased following treatment with a drug (IPD-1151T) that altered the IC/PBS phenotype and symptoms in these IC/PBS patients, suggesting that IL-4 may provide a desired anti-inflammatory effect that can alter the overall cytokine profile and subsequent recruitment of T-cells and mast cells. However, because of the pleiotropic effects of IL-4 on the whole body immune system and the relatively short half-life of the peptide, systemic IL-4 administration would not be suitable for treatment of inflammatory

pain conditions in humans, suggesting an alternative strategy for delivering IL-4 as a therapeutic is necessary.

Previous studies showed the efficacy of gene delivery using replication-deficient HSV vectors, which encode therapeutic genes such as preproenkephalin or glutamic acid decarboxylase (GABA synthesis enzyme), to target organs as an efficient way to administer short half-life peptides.[10-14] It has also been demonstrated that HSV vectors injected to the bladder wall can be transduce to L6-S1 DRG via afferent nerve termini that innervate the bladder and express reporter or therapeutic gene products in the bladder and DRG for up to several weeks in rats.[15,16] We have also reported that rats treated with HSV vectors expressing preproenkephalin showed reductions in bladder overactivity and nociceptive behavior induced by capsaicin.[15] However, to date, the effect of anti-inflammatory therapy using HSV vectors expressing IL-4 has not been studied in bladder pain and overactivity animal models although a previous study showed that HSV-based IL-4 gene therapy was effective in a rat model of neuropathic pain.[14]

Therefore, we examined the effects of localized and targeted gene therapy using replication-deficient HSV vectors expressing murine IL-4 on bladder overactivity and nociceptive behavior induced by intravesical application of resiniferatoxin (RTx), a

potent analog of capsaicin, in rats.

RESULTS

Histochemistry of HSV Vector-Mediated Transgene Expression

One week after injection of the HSV virus vector SHZ expressing β-galactosidase (HSV-lacZ) (Figure 1), we observed histochemical reactivity for β-galactosidase (*lac*Z) in both the bladder (data not shown) and L6 DRG, which contain bladder afferent neurons, (Figure 2). In DRG sections, small and medium size cell bodies of afferent neurons stained positively for *lac*Z expression as we similarly found in our previous studies using the same SHZ vector. [15,17,18] We also previously demonstrated that *lac*Z expression is observed mainly in the muscle layer of the bladder after bladder wall inoculation of the same SHZ vector.[15,17,18] These results indicate that HSV vectors can be transported to bladder afferent pathways after their bladder wall inoculation.

Quantitation of HSV Vector-Mediated IL-4 Expression by ELISA

HSV vector-mediated IL-4 upregulation in the bladder and L6 DRG were evaluated using ELISA. Two week after viral vector injection, elevated expression of murine IL-4 protein was detected in L6 DRG and the bladder of rats injected with the HSV vector S4IL4 expressing IL-4 (HSV-IL4) (Figure 1) by ELISA (Figure 3). In the bladder, a significant difference was detected between the SHZ (n=4) and S4IL4 (n=4) animal

groups (0.01 ± 0.01 vs. 5.62 ± 1.64 pg/mgTP). In L6 DRG of S4IL4-injected rats, there was 13.27 ± 3.76 pg/mgTP of murine IL-4 while no murine IL-4 was detected in L6 DRG of SHZ-injected rats. These results indicate that the IL-4 protein levels were increased after IL-4-expressing HSV vector inoculation in the bladder.

Cystometric Assessment of Bladder Function

Figure 4 shows representative traces of intravesical pressure during cystometry of the SHZ and S4IL4 animal groups (one week after viral injection). There were no significant difference in intercontraction interval (ICI) between the S4IL4 (n=9) and SHZ groups (n=9) during 2 hours of saline infusion ($p=0.34$), indicating that S4IL4 did not affect normal bladder activity. However, expression of IL-4 from the S4IL4-treated rats suppressed bladder overactivity induced by nociceptive stimuli in the bladder. After RTx administration (Figure 5), the SHZ-injected control vector group showed a significant reduction in the ICI ($p<0.05$) while the ICI of S4IL4-treated rats was not significantly altered ($p=0.42$). These results indicate that IL-4 gene transfer treatment can suppress bladder overactivity induced by RTx-induced bladder irritation without affecting baseline bladder activity.

Nociceptive Behavior in Response to Pain Induced by RTx.

We evaluated the effects of S4IL4 treatment two weeks after the bladder injection in order to examine whether the effects of IL4 gene transfer last more than one week, as consistent with HSV vector-mediated transgene expression that lasted up to four weeks.[15,19] We have previously established a methodology for examining the behavior of rats in response to painful stimuli such as capsaicin or RTx when administered to the bladder thereby affecting the bladder afferents by measuring the number and intervals of time spent of licking or freezing behavior.[14, 20] Licking behavior after bladder RTx stimulation (Figure 6A) did not differ between S4IL4- (n=8) and SHZ-treated (n=7) groups (58.14 ± 10.77 vs. 59.38 ± 7.61). However, freezing behavior (Figure 6B) was significantly ($p<0.05$) suppressed in the S4IL4 vector-injected group by 47% compared to the SHZ-treated group (30.25 ± 4.71 vs. 57.14 ± 6.29).

HSV Vector-Mediated Expression of IL-4 Reduces Expression of IL-1β and IL2

SHZ vector-treated rats (n=4) showed significantly increased expression of IL-1β (84.42 ± 9.62 pg/mgTP) ($p<0.05$) after intravesical RTx injection compared to RTx-injected S4IL4 rats (n=4) and saline-injected sham rats (n=4) (42.12 ± 7.98 and 36.62 ± 3.00 pg/mgTP, respectively). There was no significant difference in IL-1β levels between

S4IL4 vector-injected and sham rats, indicating that IL-4 gene delivery almost completely suppressed the RTx-induced IL-1β increase in the bladder. In addition, Multiplex analyses using an automated immunoassay analyzer, Luminex 200 (Bio-Rad, Hercules, CA) also revealed that the increased expression of IL-1β and IL-2 that was seen after intravesical RTx administration (3 μM, 0.3mL for1 min) in STZ-injected rats (n=6) were prevented in S4IL4-injecetd rats (n=6) (Table 1). However, IL-6 or TNFα levels were not altered after intravesical RTx administration in either SHZ or S4IL4-injecetd rats (Table 1), indicating that increased expression of IL-6 or TNFα is not induced in this animal model of RTx-induced bladder irritation.

Assessment of inflammatory responses in the bladder

The effects of HSV vector-mediated IL-4 expression on bladder inflammatory responses were evaluated by histological analysis of hematoxylin and eosin-stained bladder specimens and myeloperoxidase (MPO) assay that can measure tissue neutrophil activity (Figure 4). There were histological changes such as submucosal edema evidenced by an increase in the mucosal layer width after intravesical RTx administration (3 μM, 0.3mL for1 min) in SHZ-treated rats; however, submucosal edema was less in S4IL4-treated rats with RTx-induced bladder irritation (Figure 8A and B). In

addition, an increase in MPO activity, which was seen after intravesical RTx administration (3 μM, 0.3mL for1 min) compared with vehicle treatment in SHZ-injected rats (n=6), was prevented in S4IL4-treated rats with RTx-induced bladder irritation (Figure 8C). These results suggest that increased IL-4 expression in S4IL4 rats can suppress bladder inflammatory responses induced by bladder irritation.

DISCUSSION

We had previously employed HSV vectors expressing products such as enkephalin[15] and GAD67[16] as means of reducing pain signaling as measured by changes in behavior and/or bladder activity when the vectors were injected into the bladder wall of rats. These prior studies dealt with products that directly altered the afferents without addressing the inflammatory component of the nociceptive process. Other studies using an HSV vector (S4IL4) expressing murine IL-4 showed that this product, when expressed in DRG afferents following vector administration to the plantar foot surface[14], enabled a reduction in nociceptive behaviors after painful insult. Our current study was initiated to examine whether the same S4IL4 vector when administered into the bladder wall of the rat would result in IL-4 expression that was capable of reducing the inflammatory component of the pain response in bladder afferent pathways.

The results of this study indicate that: (i) HSV vectors injected into the bladder wall are transported into L6 DRG neurons via afferent nerve fibers; (ii) significant increases of murine IL-4 were detected in the bladder and L6 DRG from S4IL4 vector-injected rats, (iii) in S4IL4-treated rats, the ICI was not reduced during RTx intravesical administration, which induced bladder overactivity in SHZ-treated control rats; (iv) S4IL4-injected rats showed a reduction in freezing behavior; and (v)

increased expression of IL-1β and IL-2 as well as bladder inflammatory responses such as submucosal edema and enhanced MPO activity elicited by RTx-induced bladder irritation was reduced in S4IL4-treated rats.

In this study, while freezing behavior was significantly reduced, there was no difference in licking behavior between SHZ- and S4IL4-treated rats. This is probably due to the fact that licking behavior is induced by stimulation of urethral afferents in the pudendal nerve rather than bladder afferents because previous studies including ours have demonstrated that pudendal nerve transection significantly reduces licking behavior, but not freezing behavior, induced by intravesical application of capsaicin[21] or RTx.[20] We have also shown that administration of HSV vectors expressing proenkephalin into the bladder wall suppresses freezing behavior, but not licking behavior, induced by intravesical application of capsaicin. Thus these results provide evidence that the effects of IL-4 gene transfer after S4IL4 bladder injection are limited to bladder afferent pathways, which are carried through the pelvic nerves, but not the pudendal nerves, and that neither the vector nor the transgene is having pleiotropic effects on other non-target sites.

The antinociceptive effects of HSV-IL4 treatment were found up to 2 weeks after injection in this study. In a previous study using a rat model of neuropathic pain,

the antinociceptive effect of HSV-IL4 lasted up to 4 weeks post injection, and reinoculation of HSV-IL4 re-instated the antinociceptive effect.[14] Another study using HSV vectors expressing proenkephalin also showed the presence of HSV-mediated protein expression for 4 weeks in the bladder and L6 DRG following bladder wall injection.[15] Thus, HSV-based gene delivery to bladder afferents could avoid frequent administration of short half-life peptides in a target organ, and the lack of off-target activity suggests that this type of delivery would avoid systemic side effects that have also been observed using direct delivery of the therapeutic protein(s).[22]

HSV vectors expressing IL-4 have been employed in a variety of applications where the activity of IL-4 has been shown to play a role in altering the immune response within the host. The S4IL4 vector was able to reduce both mucosal and systemic immunity in response to challenge with wild-type HSV and block zosteriform spread.[23] Other studies have been performed using a similar vector to the S4IL4 recombinant where HSV vector-mediated expression of IL-4 led to a reduction in the host immune response to wild-type virus infection, in this instance, during infection of the eye that resulted in failure of the host to clear the wild-type virus-infected cells.[23, 24] HSV vectors expressing IL-4 have also been used as a therapeutic to reduce autoimmune disease in the CNS such as experimental autoimmune encephalitis[26-30] and also Alzheimer's

disease in animal models.[31] Taken together, these studies support the use of HSV vector-mediated IL-4 expression to reduce or augment the host inflammatory response, and when these results are taken into account along with the prior study using S4IL4 injection into rodent footpad[12], they set up the basis for our current study examining IL-4 effects of bladder pain induced by RTx administration.

Inflammatory cytokines or chemokines have been reported to be involved in the development of inflammatory pain.[32-34] It has been reported that the release of cytokines or chemokines plays an important role in the neuropathic pain[34-37] and that, in the rodent models of neuropathic pain, antagonists of proinflammatory cytokine such as IL-1β reportedly reduced allodynia.[38] Meanwhile, IL-4 is recognized as one of the anti-inflammatory cytokines, which suppresses the expression of IL-1β mRNA and IL-1β protein.[39] Another study also indicated that IL-4 suppresses TNFα, IL-1 and PGE_2 in human monocytes *in vitro*.[40] IL-4 injection to the plantar inhibited the inflammatory hyperalgesia caused by carrageenin, bradykinin, and TNFα.[41] IL-4 injection into the peritoneal cavity also suppressed writhing behavior induced by zymosan or acetic acid in mice, and knee joint incapacitation induced by zymosan in rats.[42] The present study also showed that HSV vector-mediated IL-4 gene therapy suppressed increased expression of IL-1β and IL-2 as well as bladder inflammatory responses such as

submucosal edema and enhanced MPO activity elicited by RTx-mediated bladder irritation. Overall, the anti-inflammatory cytokine IL-4 seems to effectively suppress inflammation and the nociceptive responses associated with that inflammation.

It has been also demonstrated that proinflammatory cytokines such as IL-2, IL-6, IL-8 and TNFα are increased in the bladder and/or urine in IC/PBS patients.[1-4] Thus, although the etiology of IC/PBS seems to be multifactorial, neurogenic inflammation associated with cytokine/chemokine production could play an important role in the pathophysiological basis of the disease. In this study, HSV vector-mediated gene delivery of IL-4 into the bladder and bladder afferent pathways reduced the elevated levels of IL-1β and Il-2 in the bladder and suppressed bladder pain behavior as well as bladder overactivity elicited by RTx-induced chemical bladder irritation. It has also been reported that IL-4 has an anti-inflammatory effect to inhibit secretion of inflammatory cytokines including TNFα and IL-6[5-7], which are reportedly increased in the bladder of IC/PBS patients.[2-4] Thus, HSV-based IL-4 gene therapy could be an attractive modality for the treatment of IC/PBS by suppressing the inflammatory component of the disease process although the effects of HSV-based IL-4 gene therapy on inflammatory cytokines such as TNFα and IL-6 should be clarified in future studies.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats weighing 230 to 260 g were purchased from Hilltop Animal Care (Pittsburgh, PA) and used according to the experimental protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Viral Vectors

HSV vector S4IL4 expressing IL-4 (HSV-IL4), was produced as previously described[23] using Cre-lox recombination of a plasmid that contains ICP4 promoter driving the murine IL-4 gene in addition to the HCMV IE promoter-β-galactosidase expression cassette and a lox combination site into the thymidine kinase (tk) locus of d120[43], which is a replication-defective KOS strain HSV mutant, containing a deletion of both copies of the immediate-early ICP4 gene. As an isogenic control vector, we employed the SHZ (HSV-*lac*Z) virus that has the HCMV IE promoter-β-galactosidase expression cassette recombined into thymidine kinase (tk) locus of d120 by similar techniques (Figure 1).

In Vivo Viral Infection

Under pentobarbital anesthesia (30mg/kg), with lower abdominal incision, the vectors

were injected to the bladder wall using a 30-gauge 10 μl Hamilton syringe (Hamilton, Reno, NV). A total of 20 μl of viral suspension of S4IL4 or SHZ (8 x 10^4 PFU) was injected to 4 different sites of the bladder wall (5 μl at each site). After viral injection, rats were allowed to recover from anesthesia and then they were housed in an approved Biosafety level 2 (BSL-2) animal facility.

Histology

One week after bladder injection of the SHZ control vector, rats were sacrificed to harvest the bladder and L6 DRG for the detection of *lac*Z gene expression by X-gal staining. Cryostat sections (10μm) of tissues were mounted on gelatin-coated slides and fixed for 1 min in 1.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO). Then, slides were rinsed twice in phosphate-buffered saline, and incubated overnight at 37 C^o in the β-galactosidase substrate solution [5-bromo-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal; Roche, Indianapolis, IN), 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, and 5 mM $K_3Fe(CN)_6$ in phosphate-buffered saline]. In addition, the bladders from STZ or S4IL4-treated rats were removed 6 hrs after intravesical RTx administration (3 μM, 0.3mL for1 min), then fixed in an ice-cold 4% paraformaldehyde solution containing 0.21% picric acid in 0.1 M phosphate buffer (PB) for 48 hrs, and soaked overnight at 4°C in 0.1 M PB containing

increasing concentrations of sucrose (10 to 30%). The frozen tissues were cut at 10μm thickness (transverse sections) and stained with hematoxylin and eosin.

Expression of IL-4

Two weeks after HSV vector injection, rats were sacrificed to harvest the bladder and L6 DRG. Tissues were frozen on dry ice and stored at -80 C^o. The tissue was homogenized and the protein was extracted with RIPA buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The amount of murine IL-4 was determined by murine IL-4 ELISA kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN). All samples were run in duplicates. The levels of IL-4 were normalized to protein concentration, which was measured using a protein assay kit (Pierce, Rockford, Ill), and the results were expressed as picogram IL-4 per milligram of total protein (pg/mgTP).

Cystometry

One week after viral vector injection, cystometry was performed under urethane anesthesia (1.2 g/kg, s.c.). With a lower abdominal incision, a PE-50 tube (Clay Adams Division of Becton Dickinson, Parsippany, NJ) was inserted to the bladder through the bladder dome. An intravesical catheter was then connected to a pressure

transducer and to an infusion pump through a 3-way stopcock. Data was collected and analyzed with a software package (PowerLab, ADInstruments, Castle Hill NSW, Australia). After a baseline recording was established with saline infusion at 0.04mL/min, 10nM RTx was continuously administered at 0.04mL/min to the bladder to induce bladder overactivity in both S4IL4- and SHZ-injected rats (n=9 each).

Evaluation of Nociceptive Behavior

The methods for evaluation of nociceptive behavior in rats followed those described in our previous study.[19] Intravesical administration of RTx induces nociceptive behavior such as licking (lower abdominal licking) and freezing (immobility with pointing their nose toward the lower abdomen without licking).

Two weeks after S4IL4 (n=8) or SHZ (n=7) vector injection, rats were placed in metabolic cages for 2 hours for acclimation. After acclimation, 3 μM RTx (0.3 ml) was administered into the bladder through a temporary indwelling transurethral catheter and retained in the bladder for 1 min using a Bollman-type restraining device (KN-326; Natsume Seisakusho, Tokyo, Japan) to induce bladder pain behavior in a conscious condition. Thereafter, the transurethral catheter was removed, and rats were placed back in metabolic cages for evaluation. Nociceptive behaviors such as licking and

freezing were observed and scored by blinded observers for 15 min periods that were divided into 5 sec intervals. When licking or freezing occurs during each 5 sec interval, it was scored as one positive event. Thus, the maximal nociceptive score could be 180 during the 15-minute observation period.

Expression of IL-1 β in Bladder after RTx Stimulation

Two weeks after S4IL4 or SHZ vector injection (n=4 each), rats were administrated 3 μM RTx (0.3mL, 1 min) to the bladder through a transurethral catheter to induce bladder irritation. Sham rats (n=4) without viral injection were administrated 0.3mL of saline to the bladder through the transurethral catheter. Rats were sacrificed 6 hours after RTx or saline administration, and the bladder was harvested to examine the expression of IL-1β using an ELISA kit (R&D Systems, Minneapolis, MN). Thereafter, Multiplex analyses using an automated immunoassay analyzer, Luminex 200 (Bio-Rad, Hercules, CA) with a MILLIPLEX MAP Rat Cytokine/Chemokine Panel (Millipore, Billerica, MA) were also used to assay TNFα, IL-1β, IL-2, and IL-6 levels in bladder tissues of S4IL4 and SHZ-injected rats with intravesical RTx administration (3 μM, 0.3mL for1 min) (n=6 each). Bladder tissues were homogenized using RIPA lysis buffer, and homogenate was centrifuged at 10,000 rpm gravity for 10 minutes and the supernatants were stored

at -80°C until measured. The levels of cytokines were normalized to protein concentration and expressed as picogram per milligram of total protein (pg/mg TP).

MPO assay

Myeloperoxidase (MPO) concentration in bladder tissues of S4IL4 and SHZ-injected rats 6 hours after intravesical RTx administration (3 μM, 0.3mL for1 min) (n=6 each) was measured using a specific ELISA kit (Hycult biotech, Plymouth Meeting, PA). Protein concentration was determined using a Bio-Rad kit with bovine serum albumin as the standard. The MPO concentrations were standardized to tissue protein levels and expressed in ng/mg total protein.

Statistical Analysis

Data are shown as the mean ± SE. Statistical comparisons were made with the Wilcoxon signed rank test for paired data and Mann–Whitney U test for unpaired data as applicable with $p < 0.05$ considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Multiplex analyses of cytokines in bladder tissues

	SHZ		S4IL4	
	Vehicle (n=6)	RTx (n=6)	Vehicle (n=6)	RTx (n=6)
IL-1β	9.4 ± 2.2	29.5 ± 4.4**	5.8 ± 1.0	14.8 ± 3.8
IL-2	5.4 ± 2.2	9.3 ± 1.1**	5.2 ± 0.9	5.2 ± 0.7
IL-6	36.2 ± 15.7	21.2 ± 5.8	46.0 ± 1.2	19.4 ± 7.8
TNFα	6.4 ± 1.3	5.6 ± 1.4	1.8 ± 1.2	1.9 ± 0.7

Data: mean ± SEM in pg/mg total protein (T.P.) **P<0.01 (Mann–Whitney U test)

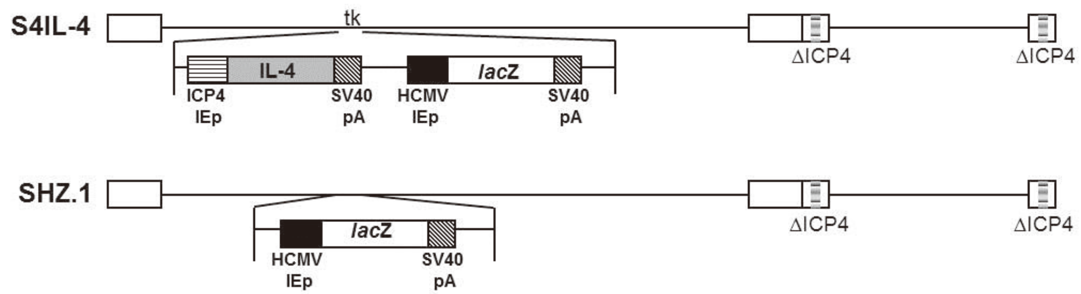

Figure 1. Schematic diagram of HSV replication-defective vectors. The control vector (SHZ) has only the E.coli *lac*Z reporter gene introduced into the tk locus of an ICP4 deleted vector while the S4IL4 vector has the murine IL-4 gene under control of the HSV IE ICP4 promoter inserted into the tk locus along with the HCMV IEp-*lac*Z reporter gene cassette.

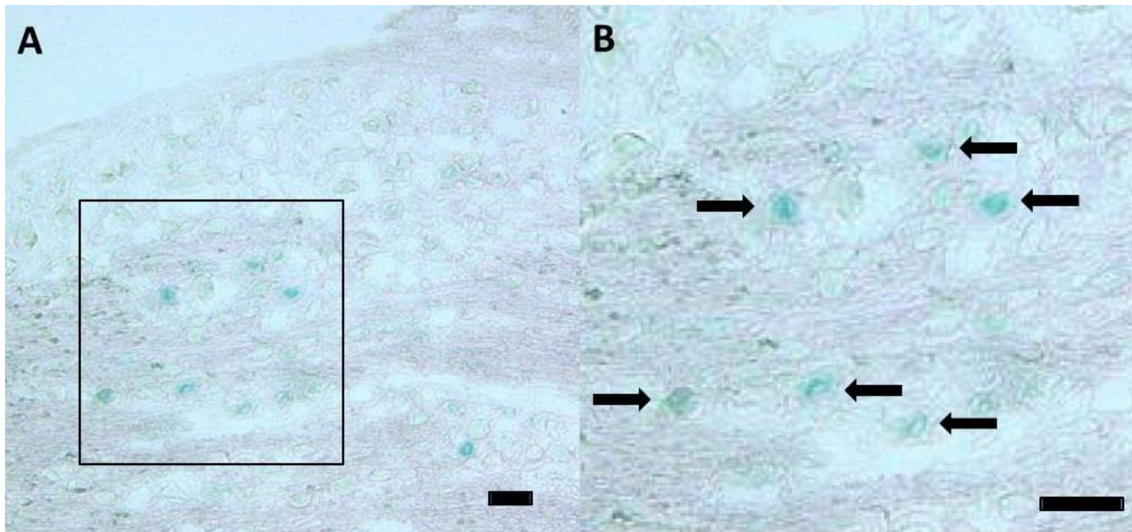

Figure 2. HSV vector transduction of DRG neurons. (A) L6 DRG section (x40). (B) L6 DRG section (x200). Panel B shows the area indicated by a rectangle in panel A with higher magnification. β-Galactosidase (*lac*Z)-positive L6 DRG neurons 1-week after SHZ vector injection into the bladder wall are indicated by arrows in panel B. Scale bars: 100 μm.

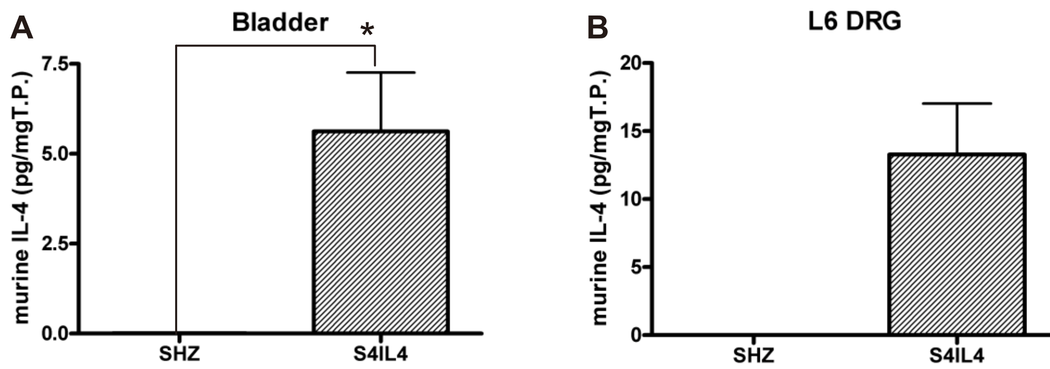

Figure 3. HSV S4IL4 vector expresses IL-4 in rat DRGs following bladder wall injection. *A,* Bladder levels of murine IL4 were significantly increased in S4IL4-treated rats than in SHZ-treated rats, in which only one rat showed a very low level of murine IL-4 in the bladder. *B,* Murine IL-4 proteins were detected in L6 DRG from the S4IL4 vector-injected group while IL-4 was not detected in L6 DRG of the SHZ control vector-injected group [(n=4 each) * $p<0.05$].

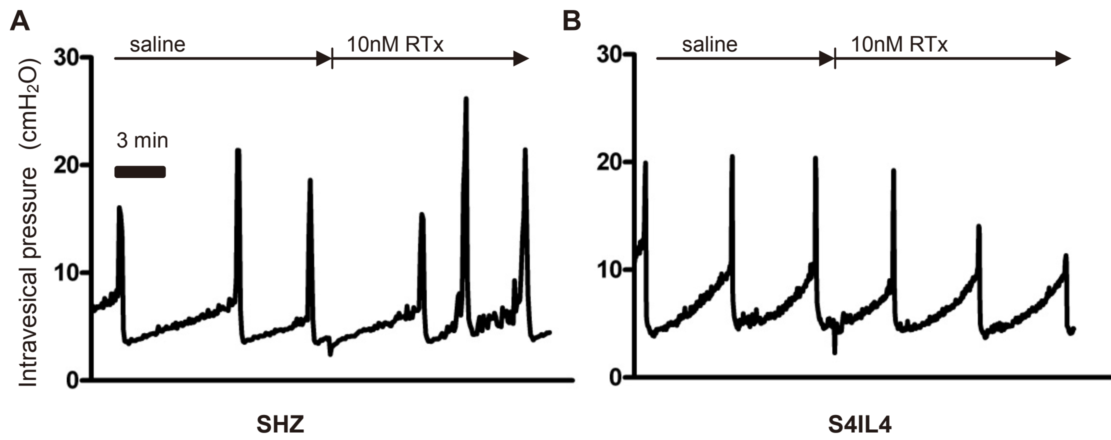

Figure 4. Representative traces of cystometry in S4IL4 verses SHZ control rats. After 10 nM RTx intravesical administration, the SHZ-treated rat (A) showed a reduction in ICI while the ICI of the S4IL4-treated rat was not altered.

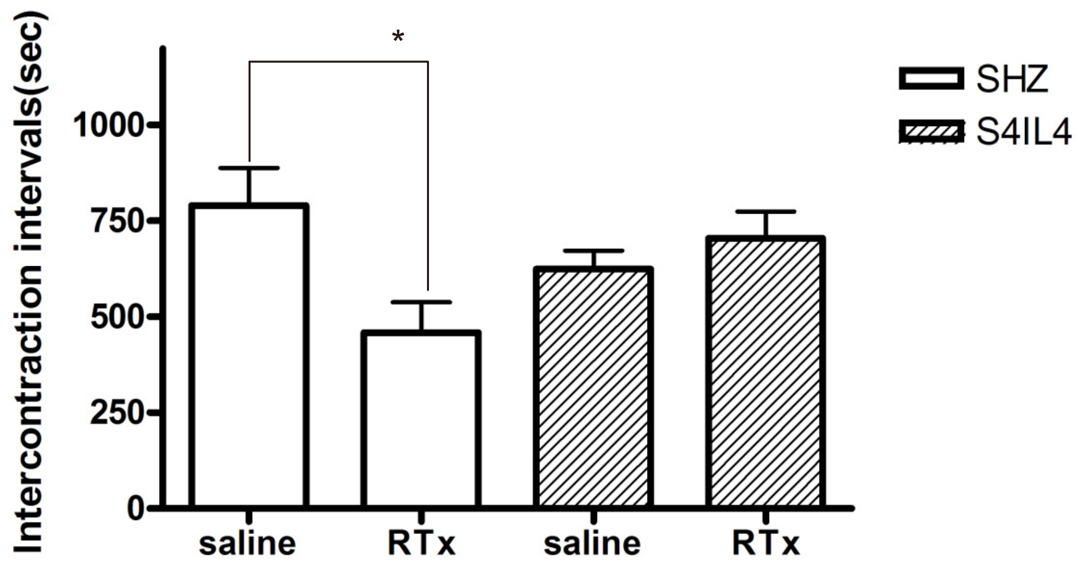

Figure 5. Effects of vector-mediated IL-4 on ICI before and after RTx infusion into the bladder. In the SHZ-treated control vector group, ICI was significantly reduced after 10nM RTx administration into the bladder. However, the RTx-induced reduction in ICI was prevented by IL-4 gene delivery in the S4IL4 group [(n=9 each) * $p<0.05$].

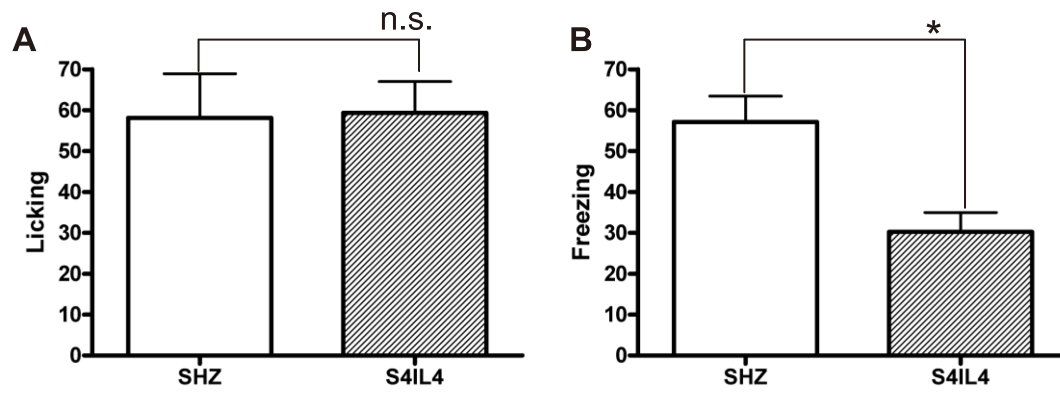

Figure 6. Effects of HSV vector-mediated IL-4 gene delivery on nociceptive behavior. *A,* Licking behavior, which is mainly related to pudendal nerve activation, was not suppressed by IL-4 ($p=0.93$). *B,* Freezing behavior, which is mainly dependent on pelvic nerve activation, was suppressed by S4IL4 [(n=7 in SHZ, n=8 in S4IL4) n.s.: not significant, * $p<0.05$].

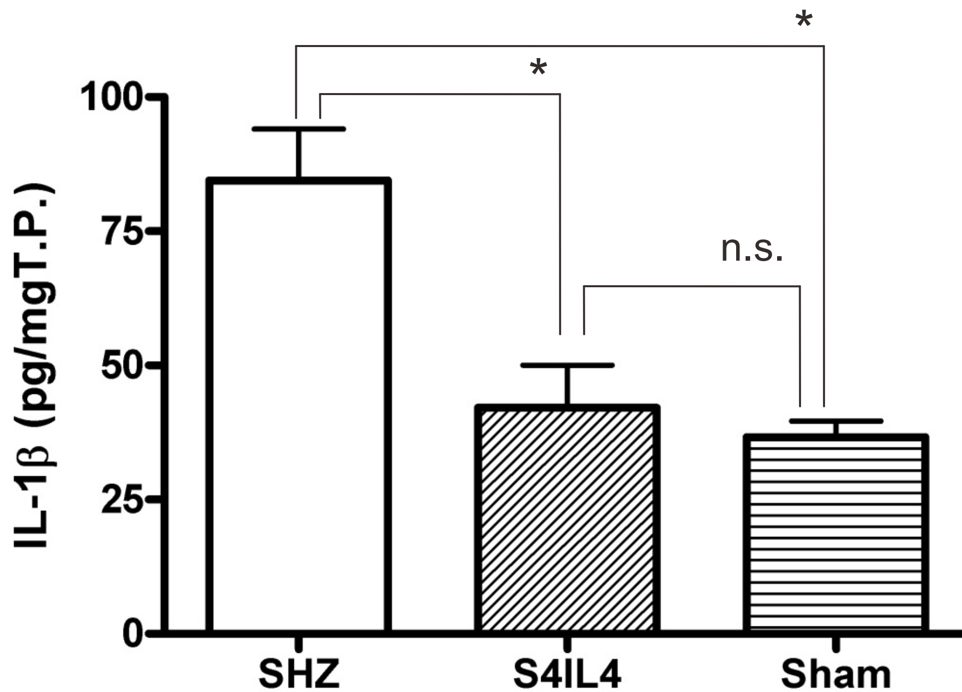

Figure 7. Quantification of IL-1β levels by ELISA in S4IL4-injected verses control vector-treated rats after bladder irritation with RTx. SHZ-treated rats showed significantly increased IL-1β expression compared to S4IL4-treated rats with RTx stimulation or HSV-uninjected sham rats without RTx. S4IL4 rats showed similar levels of IL-1β to those from RTx-untreated sham rats [(n=4 each) n.s.: not significant, * $p<0.05$].

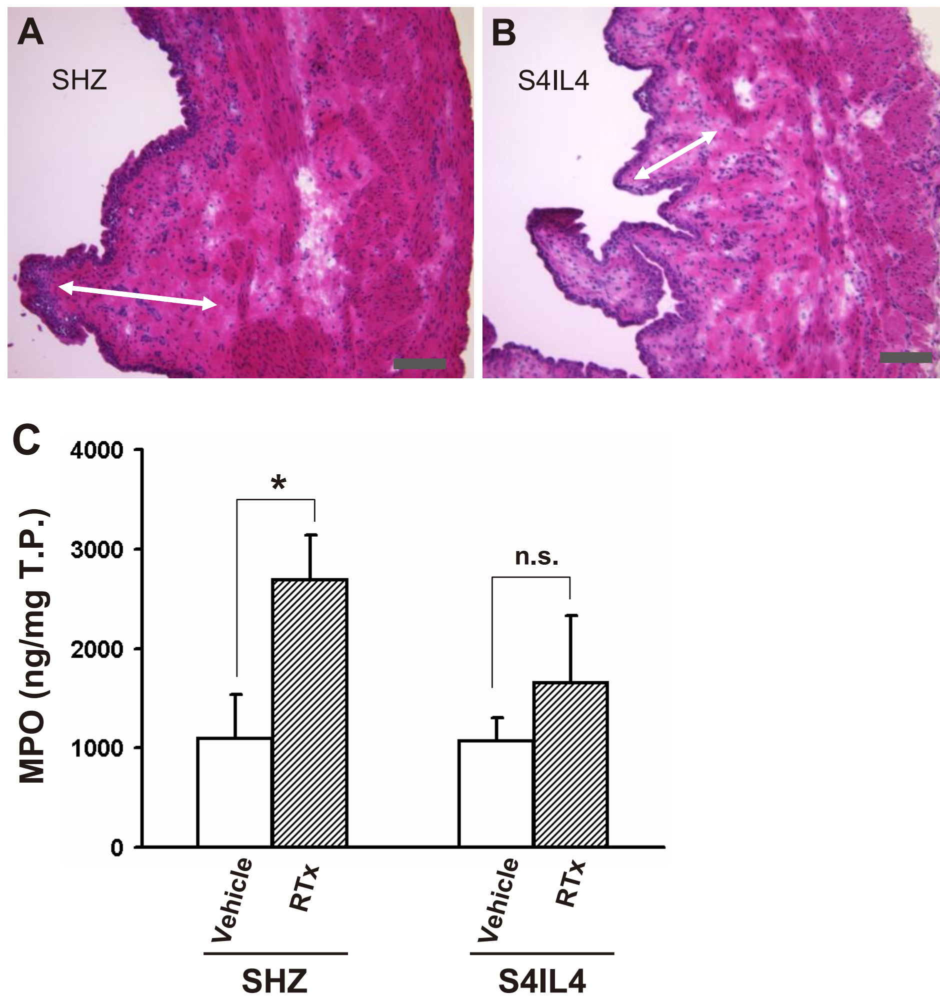

Figure 8. Histological analysis and myeloperoxidase (MPO) assay of bladder inflammatory responses. A and B: Photomicrographs of hematoxylin-eosin staining of the bladder wall 6hrs after intravesical RTx administration in SHZ (A) and S4IL4-injected rats (B). Tissue edema shown by an increase in the submucosal layer width (white arrows) was seen at a lesser degree in the S4IL4-injected rat (B) compared to the SHZ rat (A). Magnification: 40x, Scale bars: 100 μm.

C: MPO assay of bladder tissues harvested 6hrs after intravesical administration of

vehicle or RTx in SHZ and S4IL4-injected rats. A significant increase in MPO activity after RTx administration compared to vehicle was observed in STZ rats, but not in S4IL4 rats. [(n=6 each) *<0.05].