北海道公立大学法人
札幌医科大学
Sapporo Medical University
SAPPORO MEDICAL UNIVERSITY INFORMATION AND KNOWLEDGE REPOSITORY

Title 論文題目	Toll-like receptor 7 is overexpressed in the bladder of Hunner type interstitial cystitis and its activation in the mouse bladder can induce cystitis and bladder pain （Toll like receptor 7 はハンナ型間質性膀胱炎患者の膀胱粘膜組織において過剰発現し、マウス膀胱におけるその活性化は膀胱痛をともなう炎症を惹起する）
Author(s) 著　者	市原, 浩司
Degree number 学位記番号	甲第 2939 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2017-03-31
Original Article 原著論文	札幌医学雑誌　第 86 巻　第 1 号（平成 30 年 3 月）
Doc URL	
DOI	
Resource Version	

Title page

学位申請論文

Toll-like receptor 7 is overexpressed in the bladder of Hunner type interstitial cystitis and its activation in the mouse bladder can induce cystitis and bladder pain

Koji Ichihara[1, 2], Naoki Aizawa[1], Yoshiyuki Akiyama[1, 3], Jun Kamei[1, 3], Naoya Masumori[2], Karl-Erik Andersson[3], Yukio Homma[4], Yasuhiko Igawa[1*]

[1]Department of Continence Medicine, The University of Tokyo Graduate School of Medicine, Tokyo, Japan
[2]Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan
[3]Institute for Clinical Medicine, Aarhus University, Aarhus, Denmark
[4]Department of Urology, The University of Tokyo Graduate School of Medicine, Tokyo, Japan

Text pages (including pages containing figures and tables) 45
Number of Figures 8

***Corresponding author**
Yasuhiko Igawa
Department of Continence Medicine, The University of Tokyo Graduate School of Medicine,
7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, JAPAN
Tel & Fax: +81-3-5800-9792 (03-5800-9792)
E-mail: yigawa-jua@umin.ac.jp

ABSTRACT (241/250 words)

Toll-like receptor 7 (TLR7) is associated with the pathophysiology of systemic lupus erythematosus and Sjögren's syndrome, well-known diseases accompanying interstitial cystitis (IC). We studied TLR7-expression in the bladder of Hunner type IC (HIC) patients and its functional roles in bladder inflammation and nociception using mice. Bladder biopsy specimens were obtained from patients with HIC. Specimens from the non-cancerous portion of the bladder of bladder cancer patients served as controls. The specimens were examined by immunohistochemistry and RT-PCR of TLR7. Loxoribine (LX), a TLR7 agonist, was instilled in the bladder of C57BL/6N female mice, and TLR7 mRNA expression and histological changes of the bladder, bladder pain-like licking behavior, voiding behavior, cystometry, and bladder afferent nerve activities were investigated. The effects of hydroxychloroquine (HCQ), a TLR7 antagonist, on the LX-induced changes on cystometry and voiding behavior were studied. The number of TLR7 immuno-reactive cells and the mRNA expression of TLR7 were significantly increased in HIC specimens. Intravesical instillation of LX induced edema, congestion, inflammation, and significantly increased TLR7-mRNA expression in the mouse bladder. LX-instillation also

significantly increased licking behavior, voiding frequency and afferent nerve activities associated with decreased single voided volume and intercontraction interval of micturitions. HCQ reversed the LX-induced cystometric and voiding behavioral changes. TLR7 was up-regulated in the bladder mucosa of HIC patients, and activation of TLR7 in the mouse bladder induced cystitis with sensory hyperactivity of the bladder. Blocking the TLR7 pathway may be an innovative treatment target of HIC.

Keywords Toll-like receptor 7, interstitial cystitis, bladder pain syndrome, urinary bladder, inflammation

1. Introduction (353/500 words)

Interstitial cystitis (IC) is a chronic disorder characterized clinically by the presence of hypersensitive bladder symptoms (discomfort, pressure or pain in the bladder, usually associated with urinary frequency and nocturia) [9, 32]. The diagnosis can be made after exclusion of other diseases explaining symptoms. IC can be further classified by cystoscopic findings: either Hunner type interstitial cystitis (HIC) with Hunner lesions or non-Hunner type interstitial cystitis (NHIC) with mucosal bleeding after distension in the absence of Hunner lesions [9, 32]. A recent study revealed that HIC and NHIC are distinct pathological entities, with the former characterized by pancystitis, frequent clonal B-cell expansion and epithelial denudation [18]. IC is considered to be a heterogeneous entity with a variety of pathophysiological backgrounds [9, 32]. Although the pathogenic mechanisms of IC have not been determined, an autoimmune etiology is suspected based on female predominance and frequent overlap between IC and other known autoimmune disorders, such as Sjögren's syndrome, systemic lupus erythematosus (SLE), rheumatoid arthritis and ulcerative colitis [9, 32].

Toll-like receptors (TLRs) play a crucial role in the innate immune system. TLRs are essential receptors in the biological defense mechanisms against

external pathogens such as bacteria, fungus, and virus [20]. In humans, a total of 10 TLRs have been identified [2]. Among them, TLR7 recognizes single strand RNA originated from virus or bacteria, and can release inflammatory cytokines and type I interferon [10]. Since TLR7 is expressed in not only immune reactive cells but also neurons and glial cells, TLR7 is believed to be associated with the development and maintenance of inflammation and pain [13, 16, 23-24]. In addition, TLRs specifically recognize Pathogen Associated Molecular Patterns (PAMPs) *per se* [11]. Recent studies demonstrated that TLR7 contributes to developments of several autoimmune diseases, such as SLE and Sjögren syndrome by recognizing self-nucleic acid as the target [14-15]. As mentioned, such autoimmune diseases are often accompanied with IC [31]. Thus, it is hypothesized that TLR7 may participate in the pathophysiology of HIC. The aims of the present study were to evaluate TLR7 expression in the bladder tissue of patients with HIC and to examine the functional roles of TLR7 in bladder inflammation and nociception using mice.

2. Methods (1546/no limit words)

2.1 Investigations using human bladder tissue

Bladder biopsy specimens were obtained from 10 HIC patients (3 males

and 7 females, median 71 years old) and 8 patients with diagnosed bladder cancer (5 males and 3 females, median 68 years old). Biopsy specimens, consisting of urothelium and lamina propria, were obtained from the Hunner lesions and from the surrounding mucosa at the time just before hydrodistension in the HIC group. Control specimens were obtained from the non-cancerous portion of the bladder of the bladder cancer patients at the time of transurethral resection of bladder tumor. In this study, the term “background mucosa” means the bladder mucosa surrounding the Hunner lesions and which were clearly distinguished from the lesions by cystoscopy. Some of the specimens were immediately submerged in RNAlater RNA Stabilization Reagent (QIAGEN, Venlo, Netherland) and conserved at -20 ℃ to use real-time PCR (RT-PCR). Others were fixed in 4% formaldehyde and embedded in paraffin for histological examination. Study protocol was approved by the ethical committee of the University of Tokyo Hospital (no. 10581), and written informed consent was obtained from all study participants.

Immunohistochemistry

Five biopsy specimens embedded with paraffin from each of HIC and

control groups, were cut 4 μm serial sections for immunohistochemistry. Staining was performed automatically with Ventana Benchmark® XT autostainer (Ventana Medical Systems, Tucson, AZ, USA), using I-Vew DAB Universal kit. Protocol was standard CC1 and primary anti-TLR7 rabbit-monoclonal antibody diluted 1:100 (LS-B6308-50, LifeSpan Biosciences Inc., Seattle, USA) was applied for 32 minutes. Images of immunostained whole slides were digitized using BZ-X700 (Keyence, Osaka, Japan) and quantitative analysis for the number of TLR7 immuno-reactive cells was performed by the image analysis software (BZ-H3C, Keyence). For each image, both the numbers of all cell nucleus and TLR7 immuno-reactive cell nucleus of the whole specimens were automatically counted. The rate of TLR7 immuno-reactive cells in all cells was determined as % number of TLR7 immuno-reactive cells.

RT-PCR of the human bladder tissue

RNA extraction from bladder biopsy specimen was performed using RNeasy Mini Kit (QIAGEN). RNA was converted to cDNA using PrimeScript RT Master Mix (Takara-bio, Shiga, Japan). Quantitative reverse-transcription PCR was performed using Power SYBR Green PCR Master Mix and 7300 ABI Real

Time PCR System (Life Technologies, New York, USA). Messenger RNA expression was normalized relative to GAPDH. The size of PCR amplicons (base pair: bp) and the sequences of the PCR primers used are shown below: Human GAPDH (80 bp) forward: 5'-GGTGGTCTCCTCTGACTTCAACA-3', reverse: 5'-GTGGTCGTTGAGGGCAATG-3', Human TLR7 (98 bp) forward: 5'-TGGAAATTGCCCTCGTTGTT-3', reverse: 5'-GTCAGCGCATCAAAAGCATT-3'.

2.2 Animal Investigations

C57BL/6N female mice (mean weight 19.8 ± 0.1 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed under standard laboratory conditions with a 12:12-hour light: dark cycle and free access to food and water. The experimental protocol was approved by the Animal Ethics Committee (no. P-14-133) and was in line with National Institute of Health guidelines for the care and use of experimental animals.

Drugs

Loxoribine (LX), a selective TLR7 agonist, was purchased from TOCRIS

Bioscience (Ellisville, Missouri, USA) and dissolved in distilled water. Hydroxychloroquine (HCQ), a TLR7 antagonist, was purchased from Tokyo Chemical Industry (Tokyo, Japan) and dissolved in saline or 1% tracaganth gum for oral administration [8].

LX-instillation

Under an isoflurane-anesthesia, a polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was atraumatically inserted into the bladder transurethrally, and urine was drained before instillation. Next, 100 μL of LX (1.5 or 4.5 mM) or its vehicle was instilled slowly. Then, the catheter was kept for 60 minutes, after which the instilled liquid was drained. The doses of LX were determined based on a previous study [7] and our pilot investigation.

TLR7 expression on the mouse bladder

Mice were sacrificed with an overdose of pentobarbital sodium and the bladder was isolated. The bladders were manually divided into mucosal and muscle layers under stereomicroscope. Each sample was subsequently conserved in RNAlater. To examine mRNA expression changes after

LX-instillation, the mouse bladders were isolated and conserved in RNAlater at 96 hours after LX-instillation. Each conserved sample was analyzed with RT-PCR and the size of PCR amplicons and the sequences of the PCR primers used are shown below: Mouse GAPDH (86 bp) forward: 5’-AAGAGGGATGCTGCCCTTA-3’ reverse: 5’-TTTTGTCTACGGGACGAGGA-3’, Mouse TLR7 (94 bp) forward: 5’-TGATCCTGGCCTATCTCTGAC-3’, reverse: 5’-TTAGTTGCTGCGAAGAGTGC-3’.

Frequency volume (FV) measurements

Each mouse was placed in a metabolic cage (MCM/TOA-UF001-006, Mitsubishi Chemical Medience Corporation, Tokyo, Japan) for 24 hours to adapt to the environment before measurement, and voiding behavior for 24 hours was monitored as baseline. Then, the mice received intravesical LX or vehicle instillation, and were placed back into the cage. They were allowed to adapt to the environment again for 24 hours. Thereafter, voiding behavior was monitored further 3 days. Thus, in this study, day 1 to 3 as an evaluated period means 24 to 96 hours after LX or vehicle instillation. In some animals, 100 mg/kg HCQ was

orally administered in total 3 times for every 4 hours 1 hour prior to LX-instillation (4.5 mM). The dose and timing of HCQ were determined according to the hemodynamics of HCQ in mice [8] and our pilot investigation. Voiding frequency and voided volume were recorded continuously on a data acquisition program (Power Lab: AD Instruments, Sydney, NSW, Australia) for 24 hours. During recording, mice were allowed free access to water and food.

Morphological evaluation

After FV measurements, the mice were sacrificed; subsequently the bladders were isolated and opened at the dorsal side of the bladder neck along with midline towards the dome. After macroscopic evaluation, the bladders were placed into 4% formaldehyde and paraffin embedded for microscopic evaluation with hematoxylin-eosin (H-E) staining.

Bladder pain-like nociceptive behavior

Assessment of bladder pain-like behavior in mice was carried out as described previously with minor modifications [22, 30]. Each mouse was placed under a lucent plastic box and habituated to the environment for 1 hour. Then,

bladder pain-like behavior such as licking or biting of the skin of the lower abdomen close to the bladder was monitored on a video-camera for the following 30 minutes as baseline. Thereafter, the mice received intravesical LX or vehicle instillation, and bladder pain-like behavior was monitored again at several time points, which was 2, 24, 48, and 72 hours after instillation. The numbers of bladder pain-like behavior were counted by two investigators (KI, NA), and the values were averaged.

Cystometry (CMG) measurements

CMG measurements were performed in mice under a decerebrated and unanesthetized condition according to the methods reported previously [33]. In brief, under isoflurane anesthesia, a catheter (PE-50, Clay Adams) was inserted into the bladder through the dome. The head skin was removed and the skull was opened, followed by decerebration at pre-collicular position. More than two hours after the surgery, continuous CMG measurement was performed for at least 90 minutes with saline-instillation at a rate of 10 μL/min as baseline. Then, measurements were repeated with LX- or vehicle-instillation. Intravesical pressure and voided volume were recorded continuously on a data acquisition

program (PowerLab) and cystometric parameters including basal pressure (BP), threshold pressure (TP), maximum pressure (MP), intercontraction interval (ICI), and voided volume (VV) were analyzed [3]. All parameters were averaged for 60 minutes in each session. In separate animals, 0.01 mM HCQ or its vehicle was instilled into the bladder for 60 minutes after baseline measurements. Thereafter, LX (1.5 mM) with or without HCQ (0.01 mM) was instilled and CMG measurement was repeated. The dose and timing of HCQ were determined according to our pilot investigation.

***In vitro* afferent nerve activity measurements**

The procedure was performed according to a published method with minor modifications [4, 6]. Whole pelvic organs with surrounding tissues were dissected from the mouse body and placed in a recording chamber. The chamber was continuously sustained with Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 11 mM glucose) and gas (95% O_2, 5% CO_2). The urethra was catheterized using a PE-50 catheter attached to an infusion pump to enable distension of the bladder. The bladder dome was catheterized by another PE-50 catheter to enable recording

of intravesical pressure and allow evacuation of fluid. The ureters were ligated to prevent leakage from the bladder. The afferent nerves were carefully dissected into fine branches and placed into a suction electrode for recording. The electrical activity was pre-amplified with a low noise AC differential amplifier (10×) and filtered (60–5,000Hz). A final amplification (10,000×) was used and captured by a computer via a power 1401 interface and spike2 software (Cambridge Electronic Design, UK). Afferent activities were recorded during constant filling CMG with saline at a rate of 3 mL/hour, and filling continued until an intravesical pressure of 30 cmH_2O was reached. At the beginning of the experiments, recording was repeated more than 3 consecutive times at 5-minute intervals to confirm reproducibility. The last recording served as the control (before) value, subsequently LX (1.5 mM) or vehicle was repeatedly instilled 3 times with 5-minute intervals and all the 3 cycle recordings were used for evaluation. Firing rate of multi-unitary afferent activity was evaluated in relation to intravesical pressure.

Statistical analyses

All experimental data were expressed as mean ± SEM. Results were

analyzed using paired t-test, unpaired t test, Mann-Whitney`s U test, or repeated measures ANOVA followed by Dunnett's post hoc analyses. P <0.05 was considered statistically significant.

3. Results (725/no limits words)

3.1 Investigations using human bladder tissue

TLR7 expression in the human bladder

The urothelium was mostly denuded in Hunner lesions and infiltration of lymphocytes and plasma cells into the lamina propria was noted in both Hunner lesions and backgrounds of HIC bladder specimens. In immunohistochemistry, cytoplasmic expression of TLR7 was observed in the urothelial cells, and lymphocytes and plasma cells in the lamina propria of both HIC and control group (Fig. 1A). TLR7 staining intensity was relatively weak in the urothelial cells, whereas moderate to strong staining intensity was observed in lymphocytes and plasma cells. Quantitative analysis showed that the number of TLR7 immuno-reactive cells in whole specimen was significantly higher in HIC (Hunner lesions and backgrounds, $p = 0.047$ and $p = 0.028$, respectively) than the controls, while there was no significant difference between Hunner lesions and

backgrounds within the HIC group (P = 0.347) (Fig. 1B).

In RT-PCR, TLR7-mRNA expression in the bladder specimens was significantly higher in backgrounds of the HIC group than in Hunner lesions or the controls (p = 0.010 and p = 0.001, respectively), whereas there was no significant difference in its expression between Hunner lesions and the controls (p = 0.286) (Fig. 2).

3.2 Animal Investigations

TLR7-mRNA expression in the mouse bladder

TLR7-mRNA was expressed in both mucosal and muscle layers of the treatment-naïve mouse bladder, and its expression significantly increased at 96 hours after LX-instillation (1.5 or 4.5 mM: N = 4 in each) compared to baseline in the mucosal layer (1.5 mM: p = 0.023, 4.5 mM: p = 0.008), but not in the muscle layer (Fig. 3).

FV measurements

There were no significant differences in voiding frequency or voided volume among groups at baseline. In LX 1.5 mM treated animals (N = 11), no

significant differences were observed in any parameters compared with either baseline or vehicle-treated group (N = 10). In contrast, animals treated with 4.5 mM of LX (N = 11) showed increased voiding frequency throughout observation periods, and a decrease in voided volume at day 2 and day 3 compared with baseline. These changes in voiding frequency and voided volume at day 2 and day 3 from baseline were significantly greater than those of the vehicle-treated group (Fig. 4). Pretreatment with HCQ (N = 8) significantly attenuated the increased voiding frequency (day 2: $p = 0.024$; day 3: $p = 0.001$) and decreased voided volume (day 1: $p = 0.019$; day 2: $p = 0.003$; day 3: $p = 0.001$) provoked by 4.5 mM of LX-instillation (Fig. 4).

Morphological evaluation

Compared with the vehicle-treated group, macroscopic evaluation showed that the bladder walls in LX-instillation groups were thickened (Figs. 5A-C). In addition, histological evaluation with H-E staining showed inflammatory cell infiltration, edema, and capillary congestion in the sub-urothelial layer after LX-instillation (Figs. 5D-I).

Bladder pain-like licking behavior

At 2 hours after the instillation, the number of licking behavior significantly increased from the baseline in all the 3 groups, but, the increase was significantly greater in the LX-instillation groups (1.5 mM: P = 0.010; 4.5 mM: P = 0.006) than the vehicle-treated group. At day 1 (24 hours after instillation), all the 3 groups showed similar recovery of the number of licking behavior to the baseline. However, at day 2 (48 hours) in the 1.5 mM of LX-instillation group and at day 3 (72 hours) in the 4.5 mM of LX-instillation group showed a significant increase in the number of licking behavior once again compared with the vehicle-instillation group (Fig. 6).

CMG measurements

The percent changes from baseline in ICI and VV were significantly greater in the LX-instillation groups (1.5 and 4.5 mM) compare with the vehicle-instillation group, and the effect reached plateau at 1.5 mM (Fig. 7A). In comparison between HCQ and its vehicle pre-treatment groups on the percent changes of ICI and VV before and after an additional LX-instillation (1.5 mM), HCQ-pretreatment significantly attenuated the shortening ICI and decreased VV

provoked by LX (Fig. 7B). There were no significant differences in percent changes of any other parameters evaluated among the groups (data not shown).

In vitro afferent nerve activity of the pelvic nerve

During LX-instillation (1.5 mM) into the bladder, mechanosensitive multi-unit afferent nerve fiber activities significantly facilitated compared with baselines. The increased afferent nerve fiber activities in the LX-instillation group were significantly greater than those in the vehicle-instillation group (Fig. 8).

5. Discussion (962/1500 words)

In the present study, we found increased TLR7-gene expression and TLR7 immuno-reactive cells in the bladder biopsy specimens of HIC patients compared with the controls. This is, to the best of our knowledge, the first direct demonstration of the up-regulated TLR7 expressions in the HIC bladder mucosa. The number of TLR7 immuno-reactive cells was increased in both Hunner lesions and backgrounds of HIC bladders, while TLR7-mRNA expression was significantly increased only in the background but not in Hunner lesions. This discrepancy between TLR7 mRNA- and protein-expressions in the Hunner

lesions may be partly explained by the finding that the urothelium was often denuded in the lesions [18].

We also confirmed TLR7-mRNA expression in the mouse bladder, and its increased expression in the mucosal layer by intravesical instillation of LX, a TLR7 agonist. We further examined the morphological and functional changes in the mouse bladder induced by TLR7-stimulation and found that LX-instillation induced 1) acute inflammation represented as edema, congestion and inflammatory cell infiltration in the bladder suburothelial layer, 2) frequent voiding with smaller voided volume per micturition in voiding behavior measurements, and 3) increased bladder pain-like behavior. In addition, LX-instillation caused 4) shortening intercontraction interval, and 5) facilitating mechanosensitive afferent activities on cystometry. Moreover, the increased voiding frequency and decreased voided volume induced by LX were counteracted by pretreatment with HCQ, a TLR7 antagonist. These results indicate that TLR7 stimulation in the mouse bladder can induce cystitis accompanied with facilitation of inflammatory nociception and mechano-sensation of the bladder.

In a recent clinical study, involvement of TLRs in female patients with IC/bladder pain syndrome (IC/BPS) was proposed [27]. In that study, compared

to healthy controls, IC/BPS patients had higher levels of plasma inflammatory cytokines and greater cytokine-responses to TLR2 stimulation of the peripheral blood mononuclear cells. In addition, they revealed that inflammatory responses of these cells to TLR4 stimulation were associated with genitourinary pain, suggesting a role of TLR4 in painful symptoms of IC/BPS patients. Among TLRs, TLR4 has been well investigated in the lower urinary tract, because this receptor can recognize lipopolysaccharide of gram-negative bacillus, which is the most common cause for bacterial cystitis in the human [29]. Interestingly, in parallel with this ligand recognition process by TLR4, TLR7 operates to move its location from endoplasmic reticulum to endosome, where TLR7 can activate and recognize its ligands [12]. In addition, TLR7 expressing conventional dendritic cells can recognize nucleic acid originated from not only virus but also group B streptococcus [19]. Therefore, inflammation originating from bacterial infection can promote the recognition of the self-nucleic acid via TLR7 signal transduction. Furthermore, B-cells play a central role in the pathogenesis of SLE and other autoimmune diseases, and stimulation of B-cells via TLR7 leads to proliferation of autoimmune B-cells, differentiation to plasma cells, up-regulation of co-stimulatory signals, immunoglobulin production, and cytokine secretion [21].

Interestingly, a recent study using immunohistochemical quantification of infiltrating inflammatory cells revealed that the pathological feature of HIC is characterized by predominant infiltration of lympho-plasmacytic cells with frequent clonal B-cell expansion, suggesting that an abnormality in the B-cell population may be involved in the pathogenesis of HIC [18]. Given the increased expression of TLR7 in human HIC bladders as demonstrated by the present study, it is conceivable that TLR7 is a key receptor for initiation and maintenance of inflammation of HIC by modulating innate and adopted immunity.

TLR7 is expressed in nerve branches and terminals of the skin and dorsal root ganglia (DRG) neurons of mice. A recent study demonstrated that intradermal injection of a TLR7 agonist immediately increased the number of scratching, a nociceptive behavior associated with an itch [17]. In addition, an *in vitro* whole-cell patch clamp recording study in cultured mouse DRG neurons showed that the TLR7 agonist induced dose-dependent inward currents and action potentials in DRG neurons [17]. Furthermore, stimulation of cultured mouse DRG neurons with a TLR7 agonist can induce the expression and production of proinflammatory chemokines and cytokines in supernatant, which have previously been identified as mediators of pain hypersensitivity [24]. In the

present study, we found that LX-instillation into the bladder can induce bladder pain-like behavior biphasically (at 2 hours and 2-3 days after the instillation). We also found that LX-instillation immediately facilitated mechanosensitive afferent activities in an acute phase (within 2-3 hours). In rodents, at least in rats, it has been reported that C-fibers respond to the normal bladder distension as a mechanosensor [1]. Taken all together, increased nociception and mechano-sensation by intravesical instillation of TLR7 agonist may be mediated by hyperactivity of C-fibers of the bladder and DRG neurons innervating the bladder.

In the present study, we used HCQ as a TLR7 antagonist. Although HCQ has been used for an antimalarial drug, its efficacy for SLE has been well demonstrated [5, 25]. By blocking the TLR 7 and 9 in plasmacytoid dendritic cells, HCQ inhibits interferon-alpha production which plays a crucial role in SLE pathogenesis. Moreover, previous studies suggested that HCQ has a protective effect against thrombosis and increases survival in SLE patients [26, 28]. Thus, if TLR7 contributes to the pathophysiology of HIC, HCQ could become a promising therapeutic drug for HIC.

The limitations of this study should be addressed. First, the expression

of TLR7 is measured in ten HIC patients and not examined in pathologies other than HIC. Second, we did not investigate the effects of LX-instillation in the mouse bladder for more than 96 hours. Third, the association of chronic inflammation with other TLRs in the bladder is not examined.

In conclusion, TLR7 was overexpressed in the bladder of HIC patients. TLR7-activation in the mouse bladder induced cystitis and facilitation of the bladder mechano-sensory and nociceptive pathways. If valid in the pathophysiology of human HIC, the preset findings suggest that TLR7 would be a promising target for the treatment of HIC.

Acknowledgement

The present study has been supported by a Grant-in-Aid for Scientific Research (YI; Grant no. 40159588, NA; Grant no. 80595257), from the Ministry of Education, Culture, Sport, Science and Technology of the Japanese Government. Yasuho Saito provided technical advice for RT-PCR. Mitsuharu Yoshiyama provided animal experimental advice of CMG. Donna Daly provided animal experimental advice of afferent activity nerve measurement. Hiroki Ito and Rino Sugiyama provided kind advice for animal experiments.

References

1. Aizawa N, Homma Y, Igawa Y. Effects of mirabegron, a novel beta3-adrenoceptor agonist, on primary bladder afferent activity and bladder microcontractions in rats compared with the effects of oxybutynin. Eur Urol. 2012;62:1165-73. Epub 2012/09/18.

2. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801. Epub 2006/02/25.

3. Andersson KE, Soler R, Fullhase C. Rodent models for urodynamic investigation. Neurourol Urodyn. 2011;30:636-46. Epub 2011/06/11.

4. Collins VM, Daly DM, Liaskos M, McKay NG, Sellers D, Chapple C, Grundy D. OnabotulinumtoxinA significantly attenuates bladder afferent nerve firing and inhibits ATP release from the urothelium. BJU Int. 2013;112:1018-26. Epub 2013/08/14.

5. Costedoat-Chalumeau N, Dunogue B, Morel N, Le Guern V, Guettrot-Imbert G. Hydroxychloroquine: a multifaceted treatment in lupus. Presse Med. 2014;43:e167-80. Epub 2014/05/24.

6. Daly D, Rong W, Chess-Williams R, Chapple C, Grundy D. Bladder afferent sensitivity in wild-type and TRPV1 knockout mice. J Physiol.

2007;583:663-74. Epub 2007/07/14.

7. Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, Akira S, Reis e Sousa C. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol. 2003;33:827-33. Epub 2003/04/03.

8. Edwards MH, Pierangeli S, Liu X, Barker JH, Anderson G, Harris EN. Hydroxychloroquine reverses thrombogenic properties of antiphospholipid antibodies in mice. Circulation. 1997;96:4380-84. Epub 1998/01/07.

9. Homma Y, Ueda T, Tomoe H, Lin AT, Kuo HC, Lee MH, Oh SJ, Kim JC, Lee KS. Clinical guidelines for interstitial cystitis and hypersensitive bladder updated in 2015. Int J Urol. 2016;23:542-49. Epub 2016/05/25.

10. Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S, Hartmann G. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med. 2005;11:263-70. Epub 2005/02/22.

11. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216. Epub 2002/02/28.

12. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature. 2008;452:234-38. Epub 2008/02/29.

13. Lee H, Lee S, Cho IH, Lee SJ. Toll-like receptors: sensor molecules for detecting damage to the nervous system. Curr Protein Pept Sci. 2013;14:33-42. Epub 2013/02/28.

14. Lee YH, Lee HS, Choi SJ, Ji JD, Song GG. Associations between TLR polymorphisms and systemic lupus erythematosus: a systematic review and meta-analysis. Clin Exp Rheumatol. 2012;30:262-65. Epub 2012/02/14.

15. Li J, Wang X, Zhang F, Yin H. Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases. Pharmacol Ther. 2013;138:441-51. Epub 2013/03/28.

16. Liu T, Gao YJ, Ji RR. Emerging role of Toll-like receptors in the control of pain and itch. Neurosci Bull. 2012;28:131-44. Epub 2012/04/03.

17. Liu T, Xu ZZ, Park CK, Berta T, Ji RR. Toll-like receptor 7 mediates pruritus. Nat Neurosci. 2010;13:1460-62. Epub 2010/11/03.

18. Maeda D, Akiyama Y, Morikawa T, Kunita A, Ota Y, Katoh H, Niimi A, Nomiya A, Ishikawa S, Goto A, Igawa Y, Fukayama M, Homma Y. Hunner-Type

(Classic) Interstitial Cystitis: A Distinct Inflammatory Disorder Characterized by Pancystitis, with Frequent Expansion of Clonal B-Cells and Epithelial Denudation. PLoS One. 2015;10:e0143316. Epub 2015/11/21.

19. Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, Teti G, Beninati C. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol. 2009;10:587-94. Epub 2009/05/12.

20. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388:394-97. Epub 1997/07/24.

21. Meyer-Bahlburg A, Rawlings DJ. B cell autonomous TLR signaling and autoimmunity. Autoimmun Rev. 2008;7:313-16. Epub 2008/02/26.

22. Murakami-Nakayama M, Tsubota M, Hiruma S, Sekiguchi F, Matsuyama K, Kimura T, Moriyama M, Kawabata A. Polaprezinc attenuates cyclophosphamide-induced cystitis and related bladder pain in mice. J Pharmacol Sci. 2015;127:223-28. Epub 2015/03/03.

23. Nicotra L, Loram LC, Watkins LR, Hutchinson MR. Toll-like receptors in chronic pain. Exp Neurol. 2012;234:316-29. Epub 2011/10/18.

24. Qi J, Buzas K, Fan H, Cohen JI, Wang K, Mont E, Klinman D,

Oppenheim JJ, Howard OM. Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. J Immunol. 2011;186:6417-26. Epub 2011/04/26.

25. Rainsford KD, Parke AL, Clifford-Rashotte M, Kean WF. Therapy and pharmacological properties of hydroxychloroquine and chloroquine in treatment of systemic lupus erythematosus, rheumatoid arthritis and related diseases. Inflammopharmacology. 2015;23:231-69. Epub 2015/08/08.

26. Ruiz-Irastorza G, Egurbide MV, Pijoan JI, Garmendia M, Villar I, Martinez-Berriotxoa A, Erdozain JG, Aguirre C. Effect of antimalarials on thrombosis and survival in patients with systemic lupus erythematosus. Lupus. 2006;15:577-83. Epub 2006/11/04.

27. Schrepf A, O'Donnell M, Luo Y, Bradley CS, Kreder K, Lutgendorf S, Multidisciplinary Approach to the Study of Chronic Pelvic Pain Research N. Inflammation and inflammatory control in interstitial cystitis/bladder pain syndrome: Associations with painful symptoms. Pain. 2014;155:1755-61. Epub 2014/06/08.

28. Shinjo SK, Bonfa E, Wojdyla D, Borba EF, Ramirez LA, Scherbarth HR, Brenol JC, Chacon-Diaz R, Neira OJ, Berbotto GA, De La Torre IG, Acevedo-Vazquez EM, Massardo L, Barile-Fabris LA, Caeiro F, Silveira LH,

Sato EI, Buliubasich S, Alarcon GS, Pons-Estel BA, Grupo Latino Americano de Estudio del Lupus E. Antimalarial treatment may have a time-dependent effect on lupus survival: data from a multinational Latin American inception cohort. Arthritis Rheum. 2010;62:855-62. Epub 2010/02/05.

29. Song J, Abraham SN. Innate and adaptive immune responses in the urinary tract. Eur J Clin Invest. 2008;38 Suppl 2:21-8. Epub 2008/10/02.

30. Tanaka J, Yamaguchi K, Ishikura H, Tsubota M, Sekiguchi F, Seki Y, Tsujiuchi T, Murai A, Umemura T, Kawabata A. Bladder pain relief by HMGB1 neutralization and soluble thrombomodulin in mice with cyclophosphamide-induced cystitis. Neuropharmacology. 2014;79:112-18. Epub 2013/11/23.

31. van de Merwe JP. Interstitial cystitis and systemic autoimmune diseases. Nat Clin Pract Urol. 2007;4:484-91. Epub 2007/09/08.

32. van de Merwe JP, Nordling J, Bouchelouche P, Bouchelouche K, Cervigni M, Daha LK, Elneil S, Fall M, Hohlbrugger G, Irwin P, Mortensen S, van Ophoven A, Osborne JL, Peeker R, Richter B, Riedl C, Sairanen J, Tinzl M, Wyndaele JJ. Diagnostic criteria, classification, and nomenclature for painful bladder syndrome/interstitial cystitis: an ESSIC proposal. Eur Urol. 2008;53:60-7.

Epub 2007/09/29.

33. Yoshiyama M, Araki I, Kobayashi H, Zakoji H, Takeda M. Functional roles of TRPV1 channels in lower urinary tract irritated by acetic acid: in vivo evaluations of the sex difference in decerebrate unanesthetized mice. Am J Physiol Renal Physiol. 2010;298:F1351-9. Epub 2010/03/20.

Figure Legends

Fig. 1

(A) Representative TLR7 protein expressions by immunohistochemistry of the bladder mucosa in control and Hunner type interstitial cystitis (HIC) patients

The squares in the upper panels (A-C, x200) correspond to the lower panels (D-F x400). The urothelium is mostly denuded in Hunner lesions (B) and infiltration of lymphocytes and plasma cells into the lamina propria are noted in both Hunner lesions (B and E) and backgrounds (C and F) of HIC. Cytoplasmic expression of TLR7 is observed in the urothelial cells in both HIC and control group (A-C) and inflammatory cells in the lamina propria in both Hunner lesions (B and E) and backgrounds (C and F) of HIC. These cells in HIC appeared to increase in number and to be highly stained (B, C, E, and F).

(B) The % number of TLR7 immuno-reactive cells in whole specimen by quantitative image analysis

The rate of TLR7 immuno-reactive cells in all cells of the whole specimens was defined as % number of TLR7 immuno-reactive cells. *; $P < 0.05$, **; $P < 0.01$:

significant differences between groups (Mann-Whitney`s U test)

Fig. 2 TLR7-mRNA expressions in the bladder biopsy specimens of Hunner lesions and backgrounds of Hunner type interstitial cystitis (HIC) and controls

*; P <0.05, **; P <0.01: significant differences between groups (Mann-Whitney`s U test)

Fig. 3 TLR7-mRNA expressions of the mouse bladder (mucosa and muscle layer: N = 4 in each) and these changes at 96 hours after intravesical instillation of LX (1.5 or 4.5 mM: N = 4 in each)

*; P <0.05, **; P <0.01: significant differences from baseline (unpaired t test)

Fig. 4 Changes in voiding frequency and voided volume after intravesical instillation of LX (1.5 or 4.5 mM, N = 11 in each), its vehicle (N = 10), or LX 4.5 mM with oral HCQ administration (N = 8) in mice in frequency volume measurements

#; P <0.05, ##; P <0.01: significant differences from baseline (repeated measures

ANOVA followed by Dunnett's test) *; P <0.05, **; P <0.01: significant differences from vehicle-instillation group (unpaired t-test) †; P <0.05, ††; P <0.01: significant differences from LX 4.5 mM alone (unpaired t test).

Fig. 5 Representative macroscopic (A-C) and histological (D-I) findings of the mouse bladder at 96 hours after vehicle or LX-instillation (1.5 mM or 4.5 mM)

Macroscopically, the bladder wall was thickened with edema after LX 1.5 mM (B) and 4.5 mM (C) instillation compared with vehicle (A). Histological examination with H-E staining showed inflammatory cell infiltration, edema and hemorrhage (H and I) in the lamina propria after LX-instillation, but no specific histological changes after vehicle instillation (G). Square in the upper panels (D-F, x100) is corresponding to the lower panels (G-I, x400), respectively.

Fig. 6 The numbers of bladder pain-like licking behavior before and after LX (1.5 or 4.5 mM) or its vehicle-instillation (N = 6 in each group)

#; P <0.05, ##; P <0.01: significant differences from baseline (repeated measures ANOVA followed by Dunnett's test). *; P <0.05, **; P <0.01: significant

differences from vehicle-instillation group (unpaired t test).

Fig. 7 Percent changes from baseline in intercontraction intervals (ICI) and voided volume per micturition (VV) in cystometry measurements in decerebrated, unanesthetized mice after LX (o.5, 1.5 or 4.5 mM) or its vehicle-instillation (A), and percent changes in ICI and VV after LX (1.5 mM)-instillation in the presence of HCQ or its vehicle (B)

*; P <0.05, **; P <0.01: significant differences from the vehicle-instillation group (unpaired t test). †; P <0.05, ‡; P <0.05: significant difference from the LX 0.5 mM-instillation group (unpaired t test).

Fig. 8 The effects of LX on the mechanosensitive afferent nerve activities of the pelvic nerves in *in vitro* mice bladder preparations

After baseline measurements of the nerve activities, the measurements were repeated 3 times with LX 1.5 mM (N = 7, n = 37) or its vehicle (N = 7, n = 38) instillation.

#; P <0.05, ##; P <0.01: significant difference from baseline (repeated measures ANOVA followed by Dunnett's test), *; P <0.05, **; P <0.01: significant difference

between vehicle and LX 1.5mM, ǂ; P <0.05, ‡; P <0.05: significant difference from baseline (paired t test). “N” and “n” indicate the number of animals used, and number of nerve fibers recorded, respectively.

Figure 1

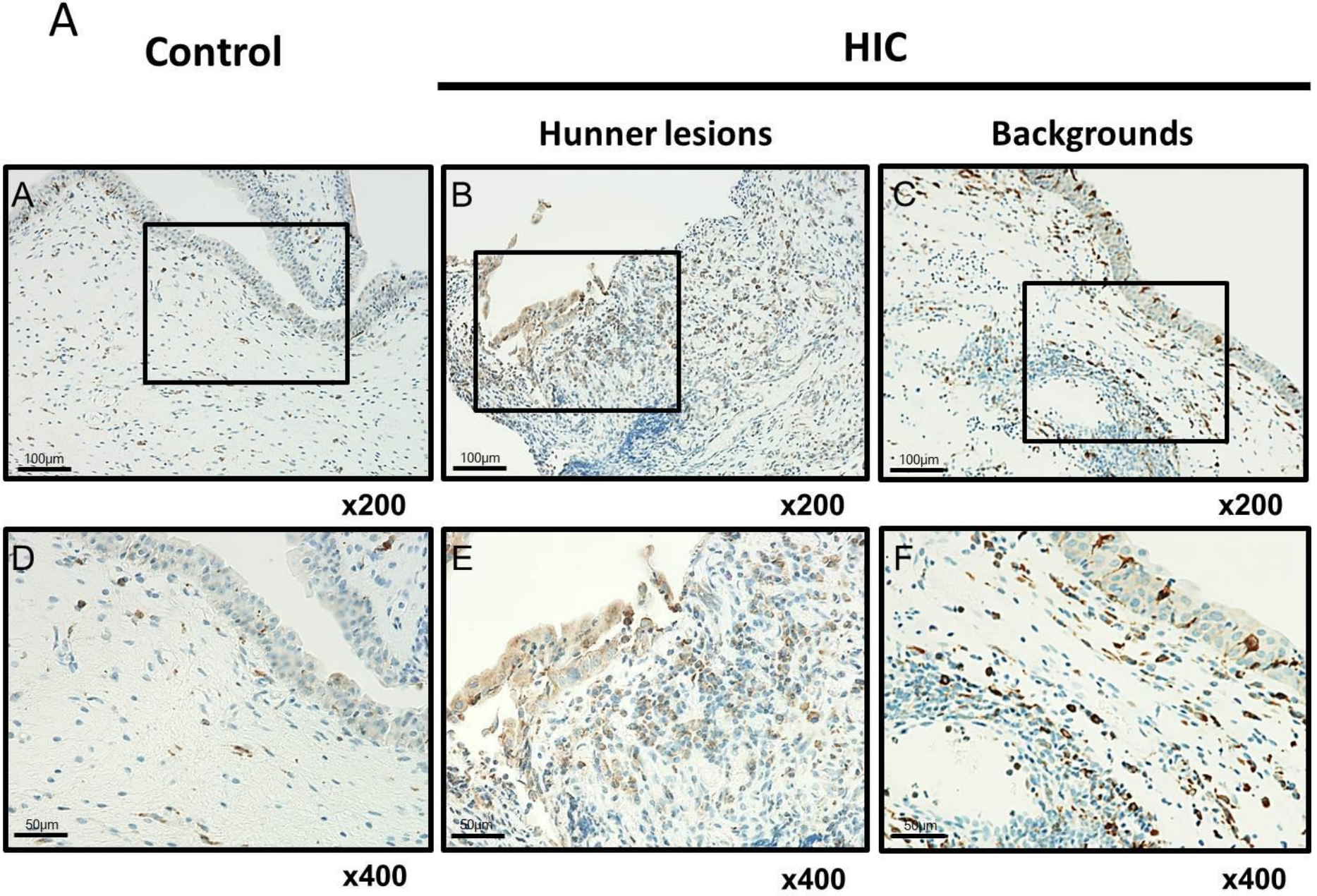

A
Control
HIC
Hunner lesions
Backgrounds
A
B
C
100μm
100μm
100μm
x200
x200
x200
D
E
F
50μm
50μm
50μm
x400
x400
x400

Figure 1

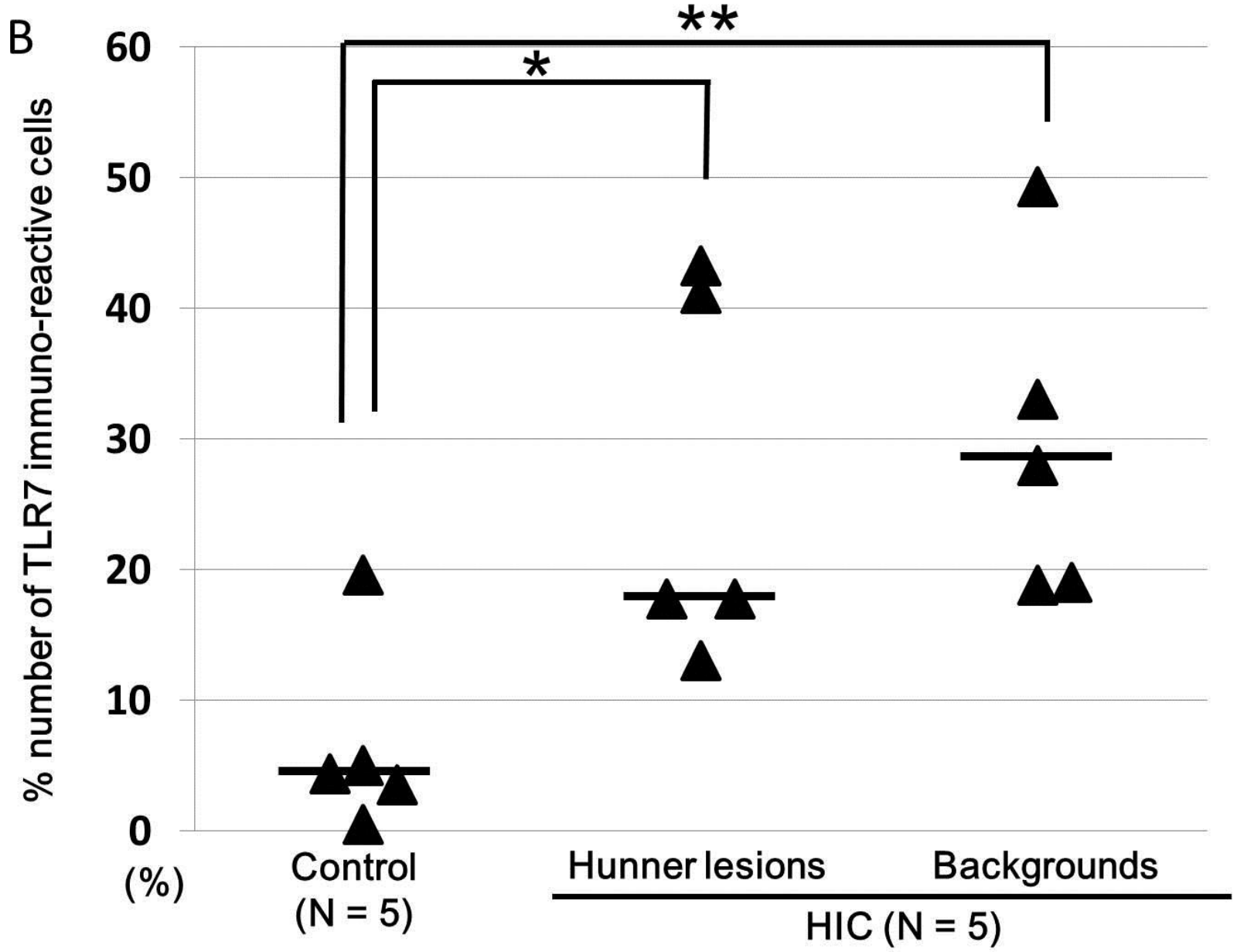

B
**
*
60
50
40
30
20
10
0
% number of TLR7 immuno-reactive cells
(%)
Control
(N = 5)
Hunner lesions
Backgrounds
HIC (N = 5)

Figure 2

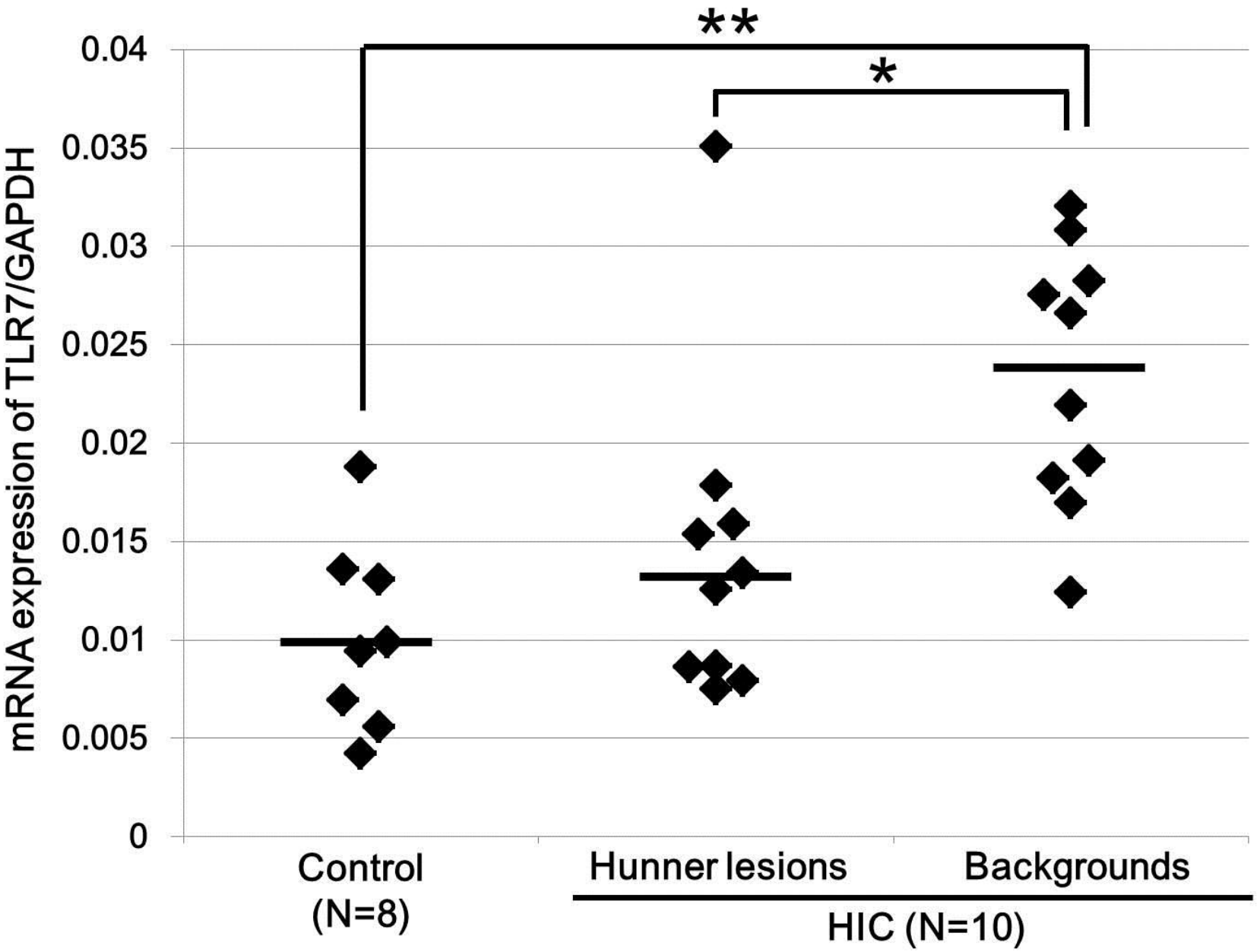

**
*
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
mRNA expression of TLR7/GAPDH
Control
(N=8)
Hunner lesions
Backgrounds
HIC (N=10)

Figure 3

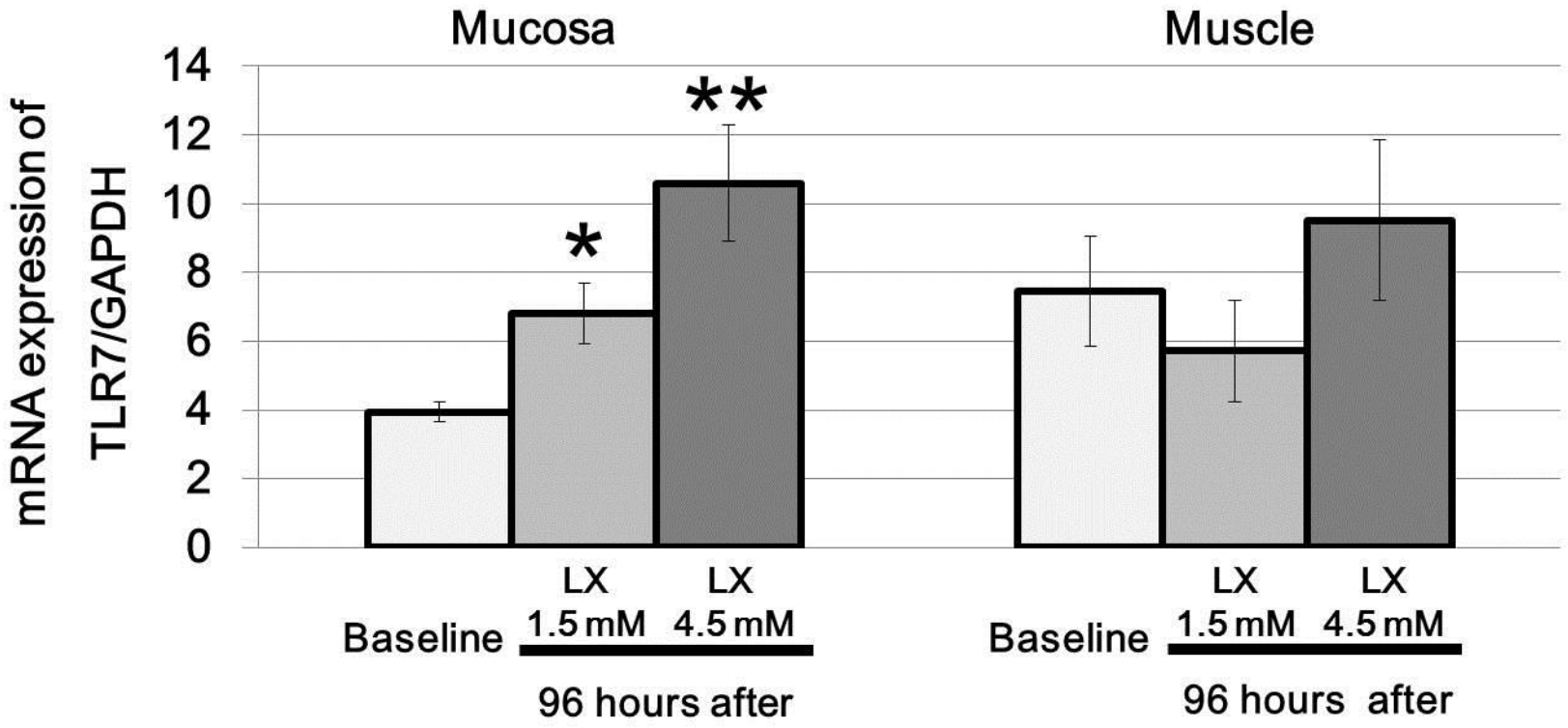

Mucosa
Muscle
mRNA expression of TLR7/GAPDH
14
12
10
8
6
4
2
0
*
**
Baseline
LX 1.5 mM
LX 4.5 mM
96 hours after
Baseline
LX 1.5 mM
LX 4.5 mM
96 hours after

Figure 4

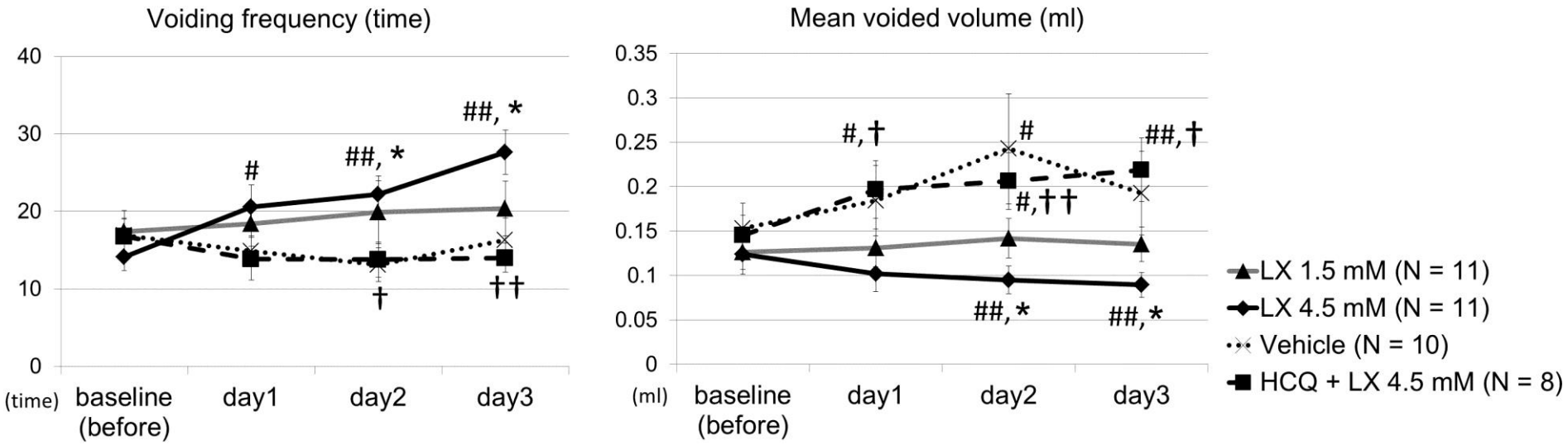

Voiding frequency (time)
40
30
20
10
0
(time)
baseline (before)
day1
day2
day3
#
##, *
†
††
Mean voided volume (ml)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(ml)
#,†
#
##,†
#,††
##,*
##,*
LX 1.5 mM (N = 11)
LX 4.5 mM (N = 11)
Vehicle (N = 10)
HCQ + LX 4.5 mM (N = 8)

Figure 5

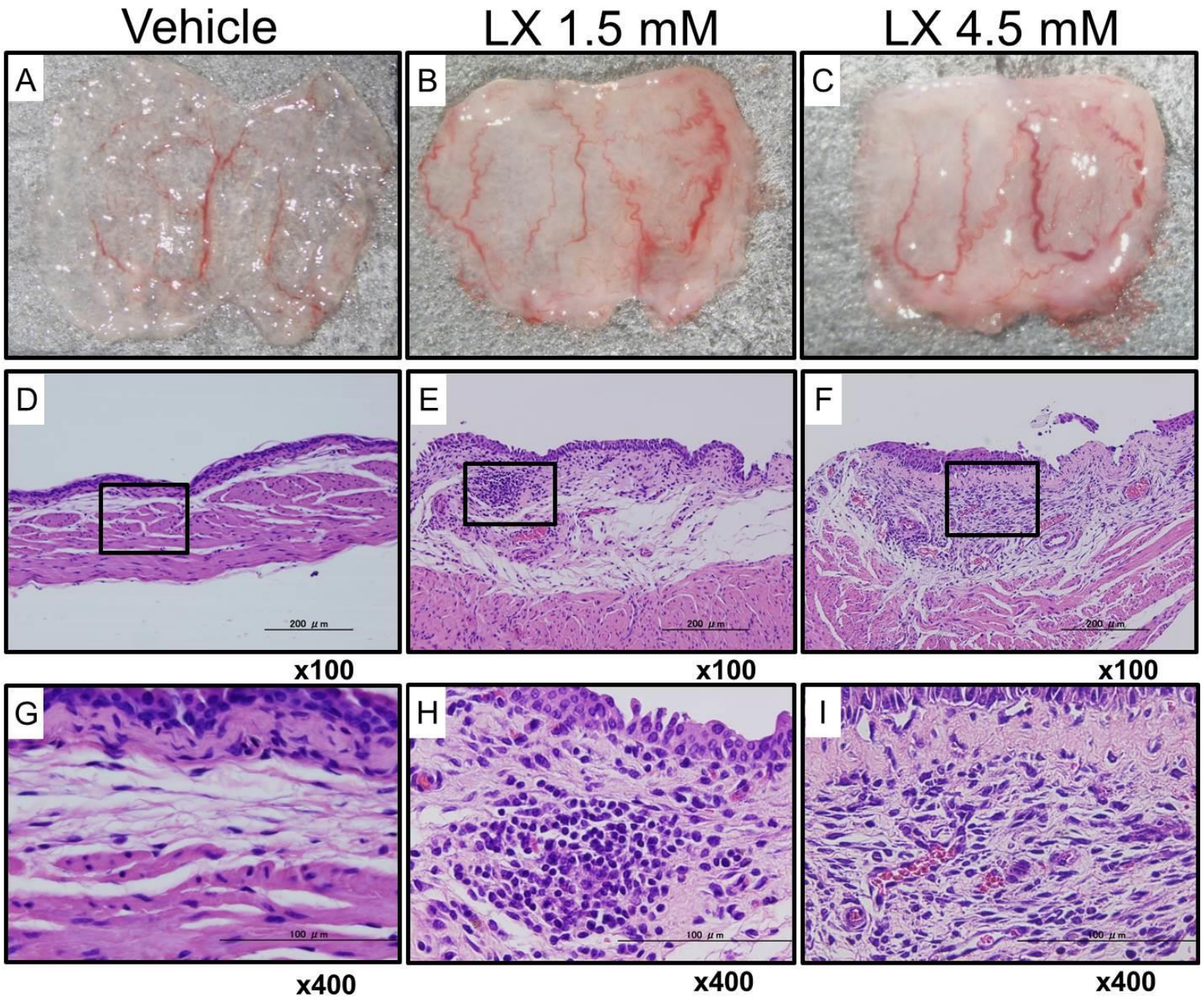

Vehicle
LX 1.5 mM
LX 4.5 mM
A
B
C
D
E
F
200 μm
200 μm
200 μm
x100
x100
x100
G
H
I
100 μm
100 μm
100 μm
x400
x400
x400

Figure 6

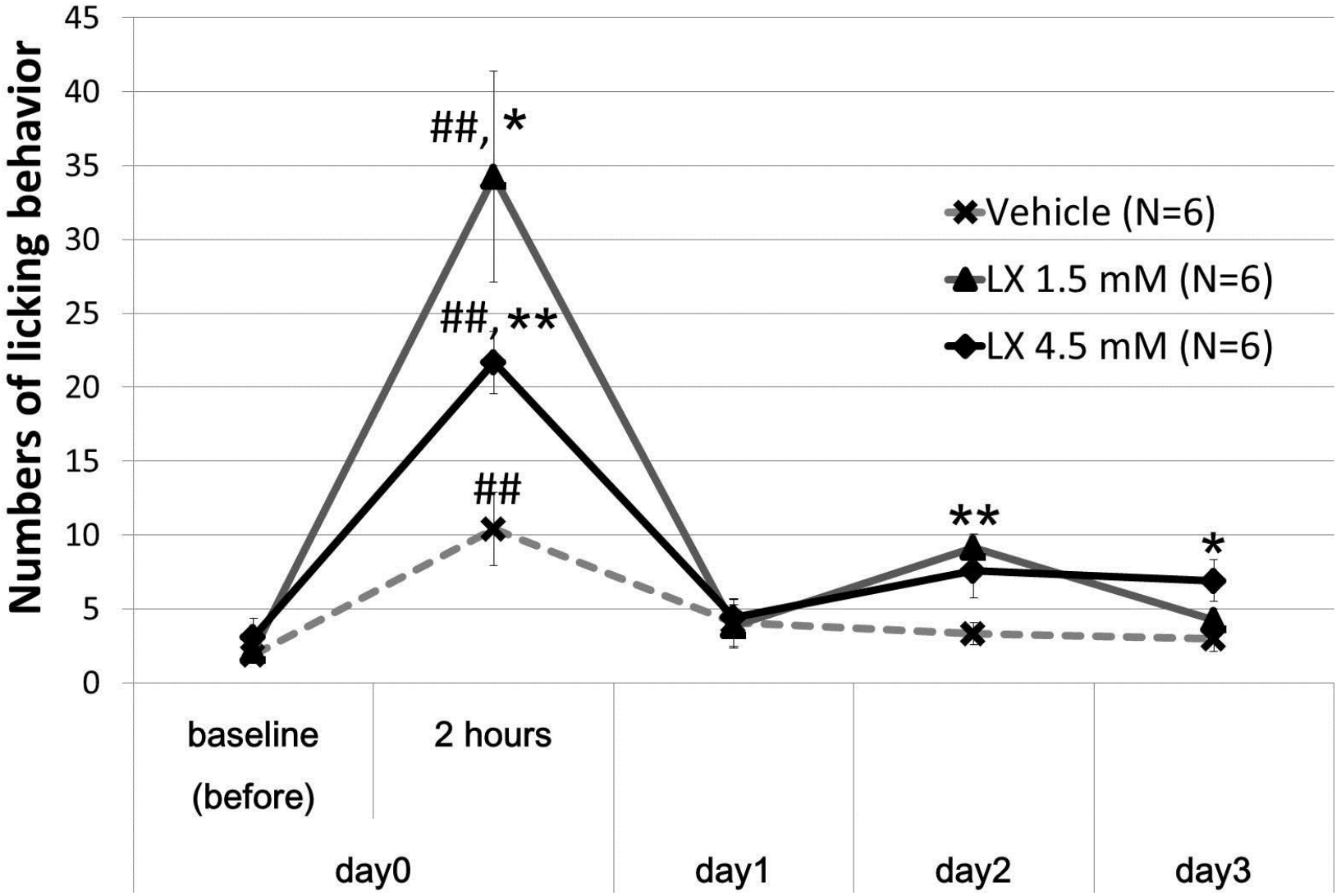
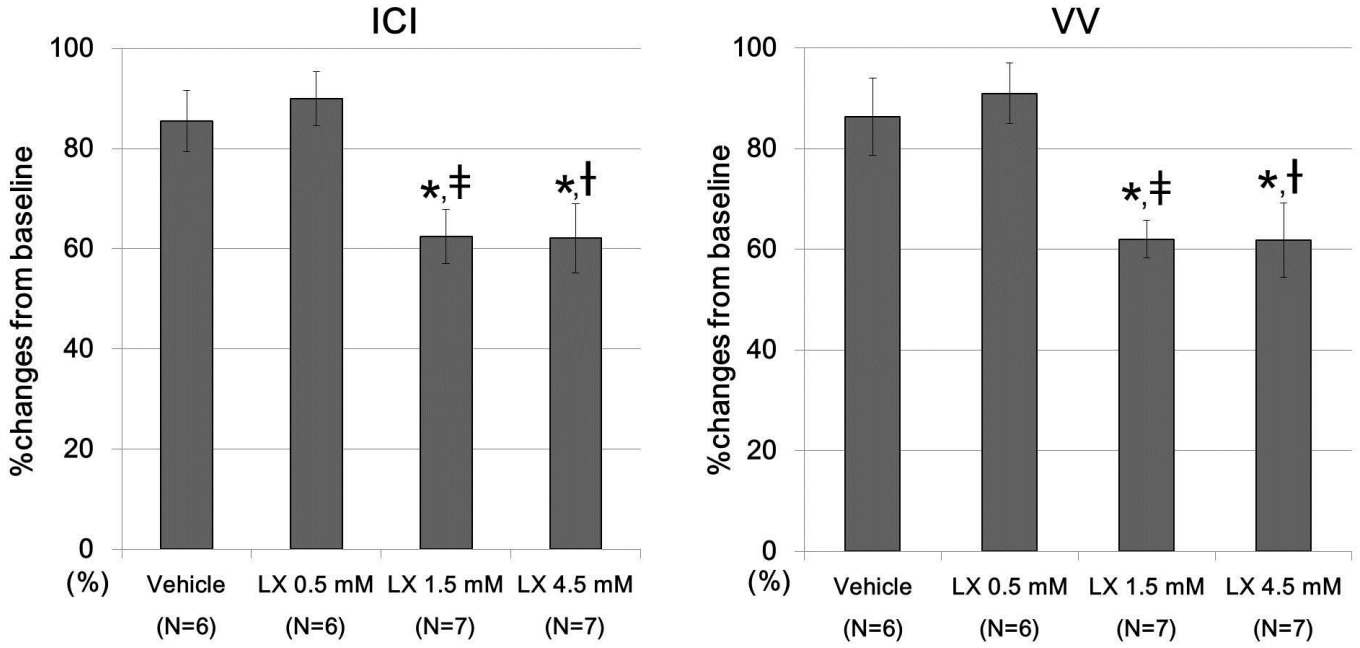

Numbers of licking behavior
45
40
35
30
25
20
15
10
5
0
##, *
##, **
##
**
*
Vehicle (N=6)
LX 1.5 mM (N=6)
LX 4.5 mM (N=6)
baseline
(before)
2 hours
day0
day1
day2
day3

Figure 7

A


ICI
VV
%changes from baseline
100
80
60
40
20
0
(%)
*,‡
*,†
Vehicle
LX 0.5 mM
LX 1.5 mM
LX 4.5 mM
(N=6)
(N=6)
(N=7)
(N=7)

B

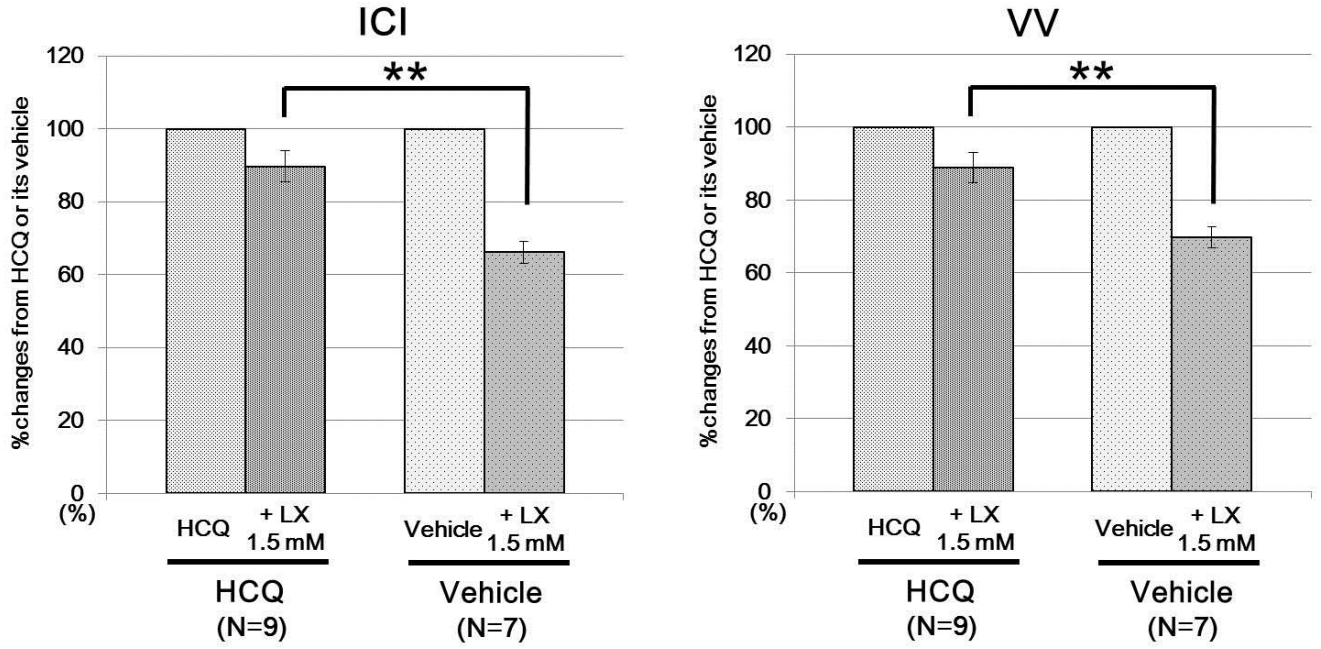
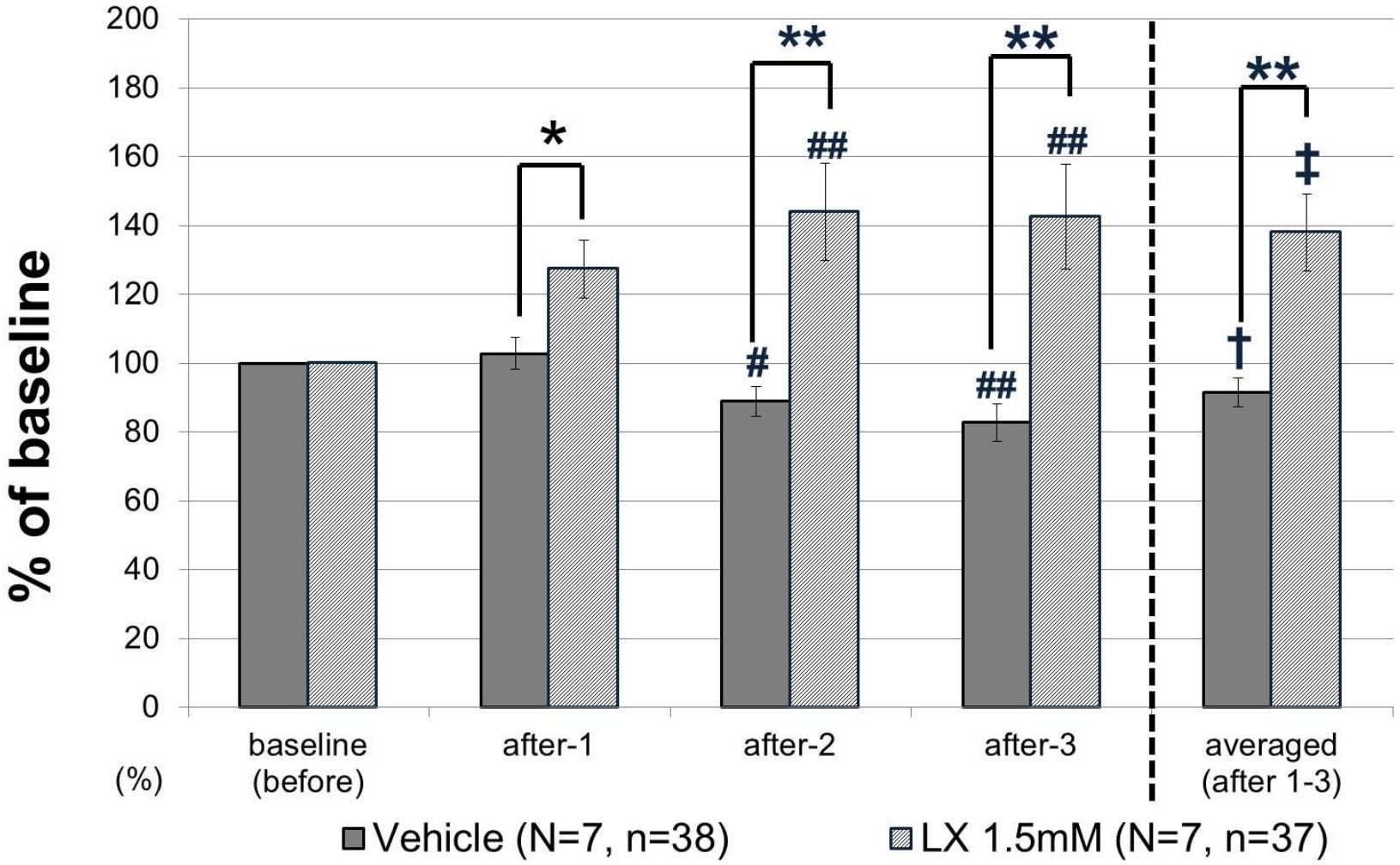

ICI
VV
%changes from HCQ or its vehicle
120
100
80
60
40
20
0
(%)
**
HCQ
+ LX 1.5 mM
Vehicle
+ LX 1.5 mM
HCQ
(N=9)
Vehicle
(N=7)

Figure 7


% of baseline
200
180
160
140
120
100
80
60
40
20
0
(%)
baseline (before)
after-1
after-2
after-3
averaged (after 1-3)
*
**
**
**
#
##
##
##
†
‡
Vehicle (N=7, n=38)
LX 1.5mM (N=7, n=37)