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Brief Communication

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Pathophysiological Role of TLR4 in Chronic Relapsing Itch Induced by Subcutaneous Capsaicin Injection in Neonatal Rats

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ABSTRACT

Despite the high prevalence of chronic dermatitis and the accompanied intractable itch, therapeutics that specifically target itching have low efficacy. Increasing evidence suggests that TLRs contribute to immune activation and neural sensitization; however, their roles in chronic itch remain elusive. Here, we show that the RBL-2H3 mast cell line expresses TLR4 and that treatment with a TLR4 antagonist opposes the LPS dependent increase in mRNA levels of Th2 and innate cytokines. The pathological role of TLR4 activation in itching was studied in neonate rats that developed chronic itch due to neuronal damage after receiving subcutaneous capsaicin injections. Treatment with a TLR4 antagonist protected these rats with chronic itch against scratching behavior and chronic dermatitis. TLR4 antagonist treatment also restored the density of cutaneous nerve fibers and inhibited the histopathological changes that are associated with mast cell activation after capsaicin injection. Additionally, the expression of IL-1β, IL-4, IL-5, IL-10, and IL-13 mRNA in the lesional skin decreased after TLR4 antagonist treatment. Based on these data, we propose that inhibiting TLR4 alleviated itch in a rat model of chronic relapsing itch, and the reduction in the itch was associated with TLR4 signaling in mast cells and nerve fibers.

Keywords: Itching; Pruritus; Capsaicin; Mast cells; Toll-like receptor 4; Th2 cells

INTRODUCTION

Itch is defined as an unpleasant sensation that induces an urge to scratch (1). Chronic itch (pruritus) increases the susceptibility to secondary infections (2), and effective therapeutics against chronic itch are ineffective. Understanding the molecular basis of pruritus is hampered by its complex mechanism. A number of molecules, such as serotonin, opioids, prostaglandins, and neuropeptides, are suggested to be itch mediators; consequently, selective serotonin receptor inhibitors or opioid antagonists have been shown to mitigate pruritus (1,3-5).

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Conflicts of Interest
The authors have no conflicts of interest to declare.

Abbreviations
PRR, pattern recognition receptor; TEWL, transepidermal water loss.

Author Contributions
Conceptualization: Kim HJ, Na HS; Data curation, Investigation, and Formal analysis: Kim HJ, Na HS, Jung Y; Investigation: Lee EH, Lim YH, Jeong D; Funding acquisition: Kim HJ, Jung Y; Supervision: Na HS, Jung Y; Writing - original draft: Kim HJ, Na HS; Writing - review & editing: Kim HJ, Jung Y.

TLRs are pattern recognition receptors (PRRs) that initiate innate immune responses by recognizing highly conserved molecular structures called pathogen-associated molecular patterns that are shared by a wide range of pathogens (6). Host cell-derived endogenous molecules released after tissue and nerve damage can induce chronic pain and itch by activating TLRs (7-11). These danger-associated molecular patterns, which consist of more than 20 endogenous molecules, such as Ags and DNA released from damaged cells, have been reported to stimulate TLRs, particularly TLR2- or TLR4-dependent pathways (7,8,10-12). Following tissue insult and nerve injury, TLRs activate microglia and astrocytes, and the resultant pro-inflammatory cytokines in the spinal cord lead to the development and maintenance of inflammatory and neuropathic pain (7). Taking into account the close relationship between chronic pain and pruritus, it is plausible that TLRs play a role in chronic pruritus. However, the roles of TLRs, which are expressed by various cutaneous immune cells, in chronic pruritus still need to be elucidated.

Mast cells are the central effectors and regulatory cells in Th2-dependent immune responses (13,14). The binding of allergens to IgE results in the cross-linking of high-affinity FcεRI receptor on mast cells, a phenomenon that induces mast cell degranulation and the release of diverse preformed or newly synthesized mediators (15). Mast cells also express a number of different PRRs, including TLRs (16). TLRs do not directly induce mast cells degranulation but instead lead to the secretion of a unique spectrum of cytokines, prostaglandins, and neuropeptides in response to ligand binding (13,14,17). Additionally, prolonged exposure of mast cells to TLR ligands modulates the effector responses of these cells and primes them for increased release of several inflammatory mediators upon subsequent activation by IgE receptors (13,18). Although mast cells are widely distributed in tissues and are present in close proximity to sensory nerve fibers (15), the precise role of inflammatory mediators released by mast cells in mediating itch remains unclear.

We hypothesized that molecules released from damaged nerve fibers could serve as endogenous ligands to activate TLR4 on mast cells. To test this hypothesis, we used a capsaicin-induced chronic itch rat model and assessed the itching behavior, mast cell activation, and production of inflammatory cytokines in neonatal capsaicin–treated rats and evaluated whether blocking TLR4 mitigates pruritus and the associated histopathological signs.

MATERIALS AND METHODS

RBL-2H3 mast cell line culture

The RBL-2H3 mast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Eagle's Modified Eagle's Medium (ATCC) containing 15% fetal bovine serum (Hyclone, Logan, UT, USA). These cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. To evaluate the effect of the TLR4 antagonist, RBL-2H3 mast cells were first treated with 10 μg/ml LPS-RS Ultrapure (Ultrapure LPS from *Rhodobacter sphaeroides*; InvivoGen, San Diego, CA, USA) for 30 min, and thereafter with 100 ng/ml LPS for 3 h.

Rat model for chronic itch and TLR4 antagonist treatment

All experiments were approved by the Korea University College of Medicine Animal Research Policies Committee (KUIACUC-2013-119). Postnatal day one Sprague-Dawley rats were obtained from Raon Bio (Yong-In, South Korea). As described previously (19), newborn male

rat pups were subcutaneously administered capsaicin (50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) within 48 h of birth. Rats were intraperitoneally injected with the TLR4 antagonist LPS-RS Ultrapure (Invivogen) at a concentration of 0.05 μg/g of body weight, twice a week until postnatal week five (beginning at 0 [W0] or 4 [W4] weeks) after capsaicin treatment.

Scratching behavior

Rat behavior was recorded for an hour using a digital video camera (HMX-QF30, Samsung, Suwon, Korea), and the number of scratches was counted. A bout of consecutive scratching strokes with the hind paw was considered as one scratch. The recordings were performed twice a week after administering the TLR4 antagonist.

Evaluation of cutaneous lesions

Skin condition at the face, ears, and back was scored as described previously (20). The sum of scores for the three regions was considered as the dermatitis score for each rat. Transepidermal water loss (TEWL) was measured on the rostral back skin using Tewameter TM 210 (Courage+Khazaka GmbH, Cologne, Germany).

Histopathology

Paraffin-embedded skin tissues were sectioned at 4 μm and stained with H&E (Sigma-Aldrich), toluidine blue (Sigma-Aldrich), and anti-PGP9.5 (#ab8189, Abcam, Cambridge, MA, USA) for histological examination. Mast cells were counted in 16 microscopic fields by Olympus BX51 microscope (Olympus, Tokyo, Japan). The intensity of PGP9.5 was quantified using *i*-SOLUTION™ (IMT i-Solution Inc., Vancouver, BC, Canada).

Real-time PCR analysis

Total RNA was extracted from the skin specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, and GAPDH mRNA was quantified by real-time PCR. PCR primer sequences are listed in **Supplementary Table 1**.

Statistics

Data are expressed as mean ± standard error of the mean unless indicated otherwise. Repeated measures ANOVA was used to analyze changes in scratching behavior, dermatitis score, and TEWL between the control and TLR4 antagonist treatment groups. Relative mRNA expression of cytokines and quantification through histological analysis were evaluated using one-way ANOVA. Significance was set at $p<0.05$.

RESULTS

RBL-2H3 mast cells express TLRs, and treatment with TLR4 antagonist reduces the expression of LPS-induced Th2 cytokines

The RBL-2H3 mast cell line expressed most TLRs, except for TLR1 and TLR11 (**Fig. 1A**). TLR4 on neuronal cells enhances histamine-mediated pruritus by potentiating TRPV1 activity (21). TLR4 on mast cells augments the production of IL-5, IL-10, and IL-13, which are key Th2 cytokines implicated in atopic dermatitis, which is characterized by long-term resistant pruritus (22,23). Considering the crucial role of mast cells in pruritus and the known role of TLR4 in the inflammation cascade and nerve-related pruritus, we hypothesized that TLR4 on mast cells could play a role in chronic relapsing pruritus and evaluated whether blocking TLR4 mitigates pruritus and inflammation. To confirm that LPS-RS Ultrapure is a potent

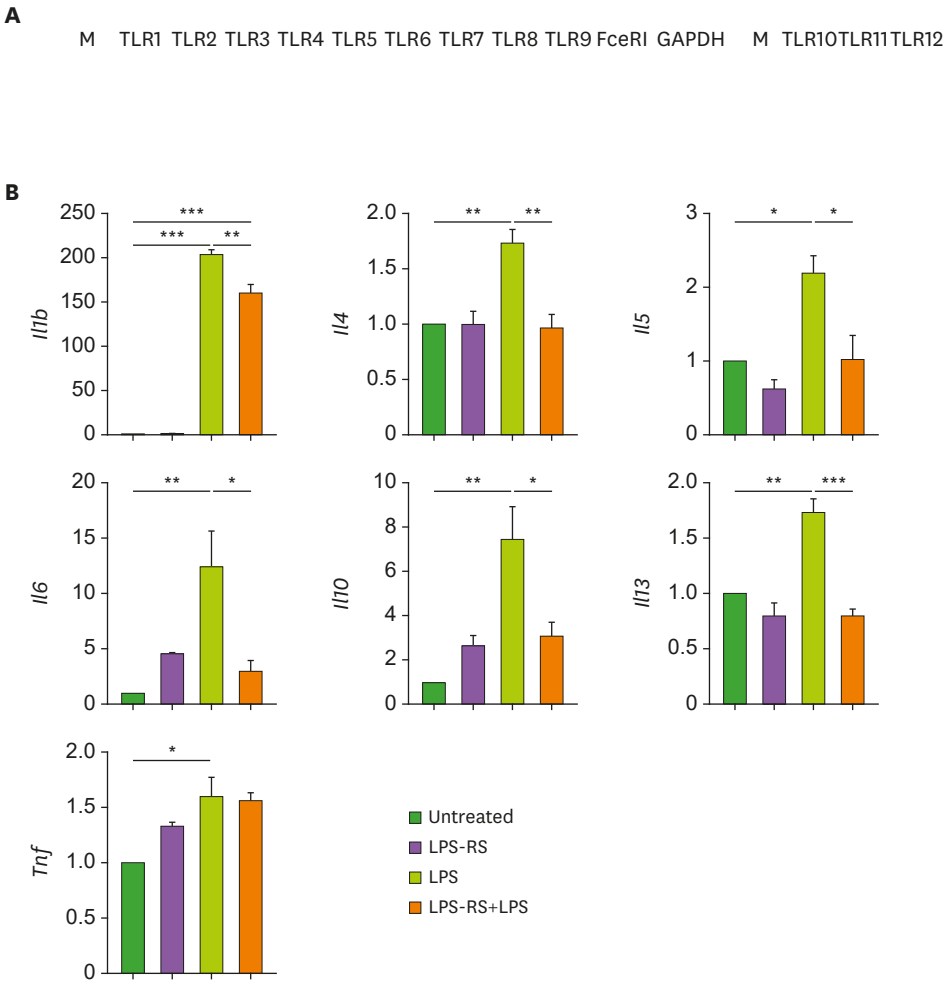

Figure 1. Assessment of TLR4 expression in RBL-2H3 mast cell line. (A) TLRs are expressed by the RBL-2H3 cells. (B) Cytokine profile after treatment with the TLR4 antagonist. The expression of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, and TNF-α mRNA was evaluated by real-time PCR after treating RBL-2H3 mast cells with 100 ng/ml TLR4 antagonist. The experiments were performed in triplicate. Graphs show mean ± SEM.
*p<0.05, **p<0.01, ***p<0.001.

TLR4 antagonist, RBL-2H3 cells were stimulated with 100 ng/ml LPS in addition to the antagonist. Treating RBL-2H3 mast cells with a TLR4 antagonist decreased LPS-induced mRNA expression of IL-1β, IL-4, IL-5, IL-10, and IL-13 (**Fig. 1B**). There was no decrease in TNF-α expression following TLR4 antagonist treatment (**Fig. 1B**).

TLR4 antagonist ameliorates scratching behavior, chronic dermatitis symptoms, and pathological changes associated with mast cell activation

Capsaicin is a neurotoxin that damages sensory neurons in spinal and cranial nerves (24). Neonatal capsaicin treatment has been successfully used to destroy the majority of C fibers in rats (25). Thus, we hypothesized that damaged nerves provide endogenous ligands that activate TLR4 on mast cells. To test this hypothesis, we administered the TLR4 antagonist LPS-RS Ultrapure to capsaicin-treated rats and evaluated the scratching behavior and skin inflammatory changes. The scratching behavior and expression of IL-31 induced by capsaicin treatment were significantly reduced by the TLR4 antagonist (**Fig. 2A** and **Supplementary Fig. 1**). This effect was not dependent on the time of injection, as evidenced by the non-

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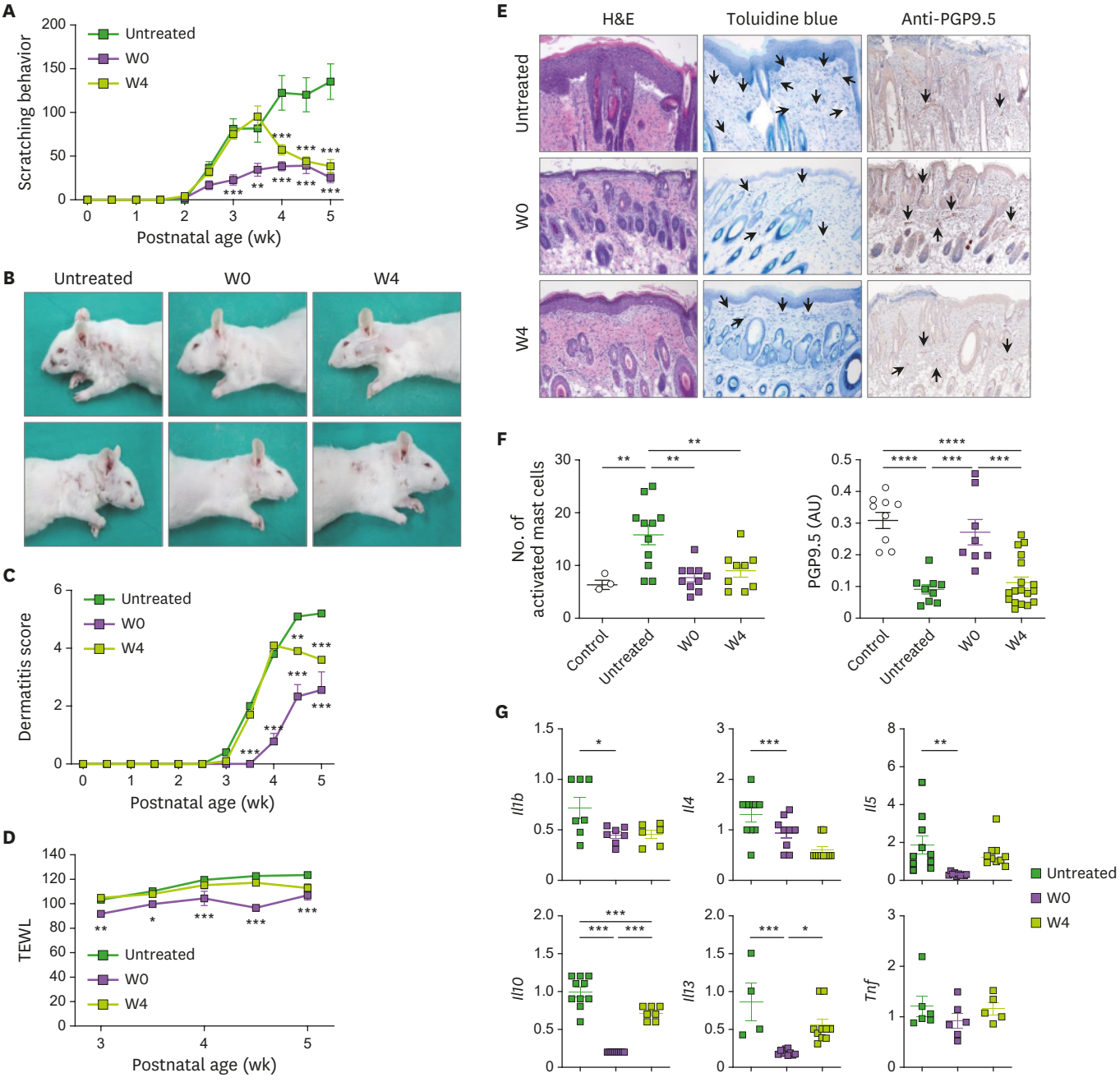

Figure 2. Effect of TLR4 antagonist on chronic itch induced by neonatal capsaicin treatment. (A) Evaluation of scratching behavior. Untreated (capsaicin injection only), W0 (treated with TLR4 antagonist immediately after capsaicin injection), and W4 (treated with TLR4 antagonist after 4 weeks of capsaicin injection). (B) Clinical manifestations. (C) The total sum of the dermatitis score obtained for the face, ear, and back. (D) Transepidermal water loss (TEWL). (E) Histopathological examination. Original magnification ×100. Arrows indicate toluidine blue-positive or PGP9.5-positive cells. (F) The number of activated mast cells (left) and quantification of PGP9.5 intensity (right) in the skin of the indicated rat. (G) Expression of the cytokine mRNA in the skins of indicated rats. (E-G) All groups were analyzed at week 5 after capsaicin treatment. W0 and W4 groups were injected with TLR4 antagonists at 0 or 4 weeks following capsaicin treatment, respectively. Graphs show the mean ±SEM.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

significant difference in the analysis of the scratching behaviors for W0 and W4 at week 5 (**Fig. 2A**). Acute inflammatory lesions, excoriation, and oozing with crust were markedly decreased in the TLR4 antagonist treatment group compared to the untreated group (**Fig. 2B**). The dermatitis score (**Fig. 2C**) and TEWL (**Fig. 2D**) were also significantly decreased after

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TLR4 antagonist treatment. Histopathological changes, such as acanthosis, lichenification, and parakeratosis with dermal inflammatory infiltrate, in capsaicin-treated lesional skin were markedly decreased by the TLR4 antagonist (**Fig. 2E**). The number of toluidine blue-positive activated mast cells was also significantly decreased by the TLR4 antagonist, without a notable difference between the W0 and W4 groups (**Fig. 2E and F**). In addition, the expression of tryptase and histamine, molecules associated with mast cell activation was also decreased by TLR4 antagonist treatment (**Supplementary Fig. 1**). As the expression of the neuronal marker PGP9.5, decreased by capsaicin treatment, was significantly upregulated only in the W0 group (**Fig. 2E, F**, and **Supplementary Fig. 2**), it is plausible to suggest that restoring damaged neurons through TLR4 antagonist requires more time than inhibiting mast cell infiltration. Additionally, treatment with the TLR4 antagonist inhibited the expression of IL-1β, IL-4, IL-5, IL-10, and IL-13 mRNA in the cutaneous lesions of capsaicin-treated rats (**Fig. 2G**). Collectively, our observations indicated that impeding TLR4 signaling after the induction of itch efficiently inhibited the histopathological and inflammatory changes in skin that are associated with TLR4 signaling due to damaged nerves and activated mast cells.

DISCUSSION

Chronic itch is an intractable symptom of a dermatological, systemic, and neurogenic disease that has a significant impact on the quality of life (26). Histamine is the most well-known pruritogen (15), and anti-histamines often fail to show significant improvements because a number of histamine-independent pruritogenic mediators are responsible for itching (27). Considering that mast cells are the primary source of pruritogens (28) and are closely associated with nerve fibers in the peripheral tissue, it may be suggested that a functional interaction between mast cells and damaged sensory nerves mediates the itch. Here, we demonstrated that blocking TLR4 inhibits the production of pro-inflammatory cytokines and mitigates pruritus and associated cutaneous inflammation induced in rats by neonatal capsaicin treatment. In this study, we observed a significant increase in the expression of genes encoding innate and Th2-type inflammatory cytokines in the LPS-stimulated mast cell line. This increase in the expression of genes encoding innate and Th2-type inflammatory cytokines was reversed by TLR4-antagonist treatment. The location of mast cells in the skin, gut, and airway causes these cells to respond to pathogens by expressing a variety of PRRs (29). Since neonatal capsaicin treatment considerably damages C-fibers (25), nerve destruction-induced activation of the PRRs on mast cells may contribute to the subsequent inflammatory cascades. However, TLRs are universally present not only on immune cells but also in a variety of cell types, including nerve cells (30). Among the PRRs, TLR4 is crucially implicated in neuroinflammation (31). Since TLR4-antagonist treatment also restored the density of cutaneous nerve fibers in capsaicin-treated chronic itch-induced rats, we suggest that TLR4 signaling in both mast cells and nerve fibers might be responsible for the pathogenesis of pruritus. Considering the scratching behavior and signs of dermatitis were still observed in rats treated with TLR4 antagonist since birth, albeit significantly decreased compared to the untreated group, it is plausible that a redundant mechanism, other than TLR4 activation, underlies chronic itch. Verifying the pathophysiological interactions between mast cells and the sensory nervous system in the induction of dermatitis and pruritus via TLR4 might help in exploring effective treatments for several dermatoses with refractory pruritus.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Primer sequences for real-time PCR

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Supplementary Figure 1

Expression of IL-31, tryptase, and histamine in the skin of untreated (capsaicin injection only) and W0 (treated with TLR4 antagonist immediately following capsaicin injection) groups

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Supplementary Figure 2

The histopathological examination of toluidine blue and PGP9.5-positive cells in the skin of healthy control

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