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Original Article

Botulinum toxin type A inhibits chronic post-thoracotomy pain through the HMGB1-mediated TLR4/NF-κB signaling pathway

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Abstract: Objective: To elucidate the analgesic role and underlying mechanism of botulinum toxin type A (BTX-A) in chronic post-thoracotomy pain (CPTP). Methods: Postoperative wound scar tissues were collected from patients with and without CPTP. Histopathologic changes were evaluated using hematoxylin-eosin (H&E) staining, and the expression levels of high mobility group box 1 (HMGB1), Toll-like receptor 4 (TLR4), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) were assessed. Spinal microglia were cultured *in vitro* to establish a cell model of CPTP. The activated microglial cells were then treated with BTX-A to evaluate its effects on substance P (SP)-induced microglia activation, HMGB1 expression, TLR4/NF-κB pathway, and inflammatory cytokine (TNF-α and IL-10) secretion. Additionally, microglia were transfected with an HMGB1 lentiviral vector to assess the regulatory role of HMGB1 on TLR4/NF-κB signaling, microglial activation, cytokine release, and the inhibitory effects of BTX-A. Results: H&E staining showed strong inflammatory cell infiltration and upregulated expression of *HMGB1*, *TLR4*, *IL-10*, and *TNF-α* in tissues from the CPTP group ($P < 0.05$). Transfection with HMGB1 lentiviral vector significantly increased the expression levels of TLR4, p-P65, and p-IκB-α in microglial cells, enhanced cell proliferation, and promoted IL-10 and TNF-α secretion. TLR4/NF-κB pathway activation positively regulated microglial activation and TNF-α and IL-10 expression. Moreover, HMGB1 overexpression attenuated the inhibitory effects of BTX-A on microglial activation. Conclusions: BTX-A may alleviate post-thoracotomy pain by downregulating the HMGB1/TLR4/NF-κB pathway, thereby reducing the secretion of inflammatory factors and inhibiting microglial activation.

Keywords: Chronic post-thoracotomy pain, botulinum toxin type A, microglia, HMGB1, inflammatory response

Introduction

Chronic postoperative pain refers to persistent or intermittent pain that develops after surgery, lasts for more than 2 months, and differs in character from any preoperative pain [1]. Chronic post-thoracotomy pain (CPTP) has garnered increasing attention due to its high incidence and unclear mechanism [2]. Epidemiologic data indicate that the incidence of CPTP ranges from 10% to 80%, making it one of the most prevalent forms of postoperative chronic pain [3]. In particular, more than 50% of patients experience CPTP within 3-6 months after surgery, significantly impairing both physical and mental recovery of patients [4]. For most patients, CPTP can lead to reduced pulmonary function, atelectasis, hypoxemia, and other complications. Moreover, severe pain triggers excessive release of catecholamines, thereby increasing cardiac afterload and myocardial oxygen consumption, and ultimately resulting in arrhythmia, myocardial dysfunction, and abnormal coagulation [5]. Therefore, elucidating the pathophysiologic mechanisms underlying CPTP is essential for early prevention and targeted treatment.

Post-thoracotomy pain often results from a "synergistic effect" caused by multiple nociceptive stimuli and involvement of multiple areas [6]. For example, during thoracotomy, tissue injury caused by skin and muscle dissection, rib traction, visceral compression, pleural incision,

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and placement of thoracic drainage tube can all activate the inflammatory cascade mediated by the intercostal, vagus, phrenic, and accessory nerves, resulting in peripheral and central sensitization and pain after thoracotomy [6, 7]. In the spinal cord of a rat CPTP model, the expression of substance P (SP), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β increased within 3-30 days after surgery. Attenuation of postoperative inflammatory responses significantly reduced the incidence of chronic postoperative pain in these models [8, 9]. These findings suggest that an early postoperative inflammatory response is crucial for the development of chronic postoperative pain.

Chronic neuropathic pain caused by peripheral nerve injury is associated with hyperreactivity of sensory neurons in the dorsal root ganglion and the spinal dorsal horn. Inhibiting the overactivation of microglia in the spinal dorsal horn has been shown to effectively alleviate neuropathic pain caused by peripheral nerve injury [10]. High mobility group box 1 (HMGB1) is a pro-inflammatory cytokine that is released by activated microglia and neurons. Elevated HMGB1 expression can activate Toll-like receptor (TLR)-4 and TLR2 on microglial membranes, leading to the activation of downstream NF-κB signaling and promoting the production of pro-inflammatory cytokines [11, 12]. Experimental animal studies have demonstrated that glial cell activation is associated with an increase in pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β [13, 14]. Blocking the release of these inflammatory cytokines can reduce inflammation and neuropathic pain [15]. Collectively, these findings suggest that modulation of spinal glial overactivation and inhibition of inflammatory cytokine release are key mechanisms for suppressing hyperalgesia and alleviating neuropathic pain caused by peripheral nerve injury.

Botulinum toxin (BTX) is a bacterial exotoxin produced during the growth and reproduction of *Clostridium botulinum*, with botulinum toxin type A (BTX-A) being the most commonly used in clinical practice [16]. BTX-A exerts its pharmacologic effects by inhibiting the release of acetylcholine from nerve endings and is widely used to reduce neuromuscular activity in neuromuscular hyperactivity disorder [17]. Recent research has revealed that BTX-A also possesses significant analgesic effect through peripheral nerve desensitization, prompting its clinical application for various chronic pain conditions [18]. The analgesic effects of BTX-A on neuropathic pain are related primarily to the modulation of microglia activity [19]. Vacca et al. [20] reported that, compared with untreated controls, BTX-A treatment significantly reduced the level of CD11b (OX-42), a marker of microglia in spinal dorsal horn and ventral horn, accompanied by marked alleviation of abnormal pain behaviors. Moreover, BTX-A has been shown to inhibit microglial activation and suppress the release of pro-inflammatory cytokines (IL-1β and IL-18), thereby exerting analgesic effects on neuropathic pain [21]. Based on these, we hypothesized that the BTX-A-induced inhibition of microglial activation may play a therapeutic role in CPTP.

In this study, tissue samples were collected from patients with CPTP to detect whether HMGB1 was differentially expressed in CPTP. In addition, a CPTP cell model was established using spinal microglia to investigate the effect and underlying mechanism of BTX-A in CPTP. A schematic illustration of the proposed pathogenic mechanism and experimental design of CPTP is presented in **Figure 1**.

Materials and methods

Sample collection

Postoperative wound scar tissues (n = 32) and adjacent normal subcutaneous tissues (n = 10) were collected from patients who underwent thoracotomy at the Affiliated Cancer Hospital of Guangzhou Medical University between January 2020 and May 2022.

Inclusion criteria: aged 24-57 years; complete preoperative clinical information and evaluation data; preoperative visual analog scale (VAS) score ≤ 1; no history of tumors, chronic inflammatory disease, chronic pain, or other related diseases before surgery. Exclusion criteria: preoperative diagnosis of ischemic heart disease or peripheral vascular disease; comorbid hypertension, diabetes, or hepatic or renal dysfunction.

When patients returned for routine follow-up or scar repair treatment at 1-3 months postoperatively, VAS scoring was performed, and tissue samples were collected. Based on postopera-

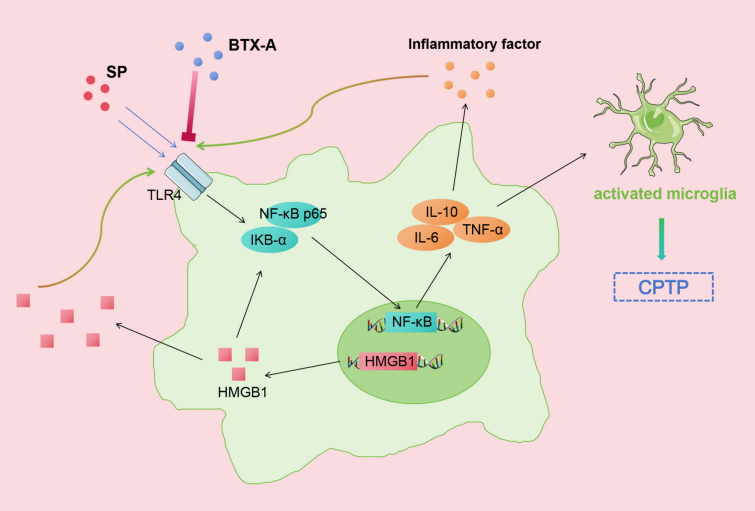

Figure 1. Schematic drawing of CPTP generation. Note: CPTP, Chronic post-thoracotomy pain.

tive VAS scores, samples were divided into a non-CPTP group (n = 19) and a CPTP group (n = 13). All collected tissue samples were stored in liquid nitrogen for subsequent analysis. This study was approved by the Medical Ethics Committee of Affiliated Cancer Hospital and Institution of Guangzhou Medical University (Approval Number: ZB2022-22B).

Cell culture

Rat spinal cord microglial cells (HAPI cells) were purchased from Wuhan Punosi Life Science and Technology Co., Ltd., and cultured in Dulbeccco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HAPI cells have been identified by STR. The HAPI cells were then cultured with 100 ng/mL SP (Med Chem Express) for 24 h to simulate the CPTP cell model [22]. BTX-A (Allergan Pharmaceuticals Ireland) was administered at a final concentration of 0.1 nmol/L. TLR4 agonist monophosphoryl lipid A (MLA) and TLR4 inhibitor (TLR4-in-C34-C2-COOH, IN-C34) were purchased from Med Chem Express.

Hematoxylin-Eosin (H&E) staining

Fresh tissue samples were fixed in 4% paraformaldehyde for more than 24 h, followed by dehydration, paraffin embedding, and sectioning. The paraffin sections were mounted on glass slides and baked at 60°C, then deparaffinized and dehydrated through graded ethanol solutions. Hematoxylin staining was performed for 3-8 min, followed by bluing color with 0.6% ammonia water. After rinsing under running water three times, the sections were counterstained with eosin for 1-3 min, dehydrated, cleared, and sealed with neutral resin. Histopathological changes were observed under a microscope.

Western blot

Paraffin-embedded tissues were first dewaxed with pretreatment reagents and then homogenized. Cultured cells were lysed using ice-cold lysis buffer to prepare total protein extracts. In brief, 100 μL of tissue homogenate or cell lysate were transferred into a 1.5-mL centrifuge tube, followed by incubation with protease digestion solution overnight. Total protein was collected by centrifugation, and its concentration was detected using a BCA kit.

After SDS-PAGE electrophoresis, the proteins were transferred to a PDVF membrane, which was then sealed with 5% skim milk at room temperature for 2 h. Next, the membrane was incubated at 4°C overnight with the following primary antibodies: HMGB1, TLR4, TNF-α, IL-10, p-P65, P65, p-IκB-α, IκB-α (all diluted 1:1000; ABclonal Technology), and β-actin (1:1000; Abcam). After washing with TBST solution three times, the film was incubated with secondary antibody (Abcam) at room temperature for 2 h. Protein bands were visualized using enhanced chemiluminescence reagents, and relative protein expression levels were quantified by normalization to β-actin.

qRT-PCR

Total RNA was extracted from cells or tissues using TRIzol reagent. In brief, 1 μg of RNA was dissolved in RNase, and the RNA concentration and purity were measured using the NanoDrop 2000 spectrophotometer. The RNA samples were then reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (TAKARA) according to the manufacturer's instructions and were either subjected to immediate PCR amplification or stored at -20°C for later use.

Table 1. PCR primer sequence

Gene	Gene ID	Primer sequence (5'-3')	
GAPDH	NM_017008.4	Forward	GCAAGGATACTGAGAGCAAGAG
		Reverse	GGATGGAATTGTGAGGGAGATG
HMGB1	NM_012963.3	Forward	GCATCTAAGCAGTATCCTCTGG
		Reverse	CTGGGCTACAAGACCCTTATG
TLR4	NM_019178.2	Forward	CGCTCTGGCATCATCTTCAT
		Reverse	CGAGGTAGGTGTTTCTGCTAAG
NF-κB P65	NM_199267.2	Forward	GGGATCCAGTGTGTGAAGAAG
		Reverse	CCTCTATGGGAACTTGAAAGGG
IKB-α	NM_001105720.2	Forward	AGTAACCTACCAGGGCTACTC
		Reverse	ATAGCTCTCCTCATCCTCACTC
TNF-α	NM_012675.3	Forward	GCAGATGGGCTGTACCTTATC
		Reverse	GAAATGGCAAATCGGCTGAC
IL-10	NM_012854.2	Forward	AGTGGAGCAGGTGAAGAATG
		Reverse	GAGTGTCACGTAGGCTTCTATG

Note: HMGB1, High mobility group box 1; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

The sequences of gene primers used are shown in **Table 1**. PCR was performed using SYBR Premix Dimmer Eraser kit (TAKARA) according to the manufacturer's instructions. The relative expression level of the target gene was calculated by $2^{-\Delta\Delta Ct}$ method, with *GAPDH* serving as the reference. PCR protocol was as follows: pre-denaturation at 95°C for 10 min and 44 cycles of reaction at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s.

Construction of lentiviral vector

The HMGB1 overexpression vector (Ov-HMGB1) and HMGB1 knockdown lentiviral vector (Sh-HMGB1-1/2/3) were constructed using pLP2 lentiviral vector (Invitrogen). The recombinant lentiviral plasmids were transfected into microglial cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of transfection, the cells were collected, and the transfection efficiency was evaluated. Sh-HMGB1-1 sequence: 5'-CCGTTATGAAAGAG-AAATGAA-3'; Sh-HMGB1-2 sequence [23]: 5'-GACCATGTCTG-CTAAAGAA-3'; Sh-HMGB1-3 sequence [24]: 5'-GATCCCG-AAGCAC-CCGGATGCTTCTTTCAAGAGAAGAAGCATCCGGGTGCTTCTTTTTTGGAAA-3'; Sh-control sequence: 5'-AGAGCCATGAAGTTAAATAAG-3'.

Immunohistochemical (IHC) staining

Paraffin-embedded tissue sections were soaked in two changes of xylene for 10 min and dewaxed with gradient alcohol, followed by treatment with Triton reagent for 20 min. The cells were uniformly covered with 10% normal rabbit serum and sealed at room temperature for 30 min to block endogenous nonspecific binding. The sections were then incubated overnight at 4°C with primary antibody solution (Abcam) in a humidified chamber. After washing with phosphate-buffered saline (PBS) three times, the sections were then incubated with secondary antibody (HRP labeled, Abcam) solution at room temperature for 50 min. Freshly prepared 3,3'-diaminobenzidine color solution was added, and staining was observed under a microscope. After color development, the sections were counterstained with hematoxylin for approximately 3 min, differentiated with ammonia water, rinsed under running water, and sealed with neutral resin. Images were captured using a microscope. Protein expression levels were semi-quantitatively evaluated based on staining intensity and the proportion of positively stained cells.

Immunofluorescence (IF) staining

Cells were cultured on coverslips and fixed with 4% paraformaldehyde. A tissue pen was used to draw a circle around the cell area, and 50-100 μL of permeabilization working solution was added to each circled area. The cells were incubated at room temperature for 20 min, followed by blocking with 10% normal rabbit serum for 30 min. Subsequently, the cells were incubated overnight at 4°C with an anti-OX-42 antibody solution (Invitrogen). After three rounds of washing with PBS, the slides were incubated with the corresponding fluorescent secondary antibody (Abcam) at room temperature for 50 min in the dark. Next, 4',6-diamidino-2-phenylindole (DAPI) staining solution was added and incubated at room temperature for 10 min to visualize cell nuclei. Finally, the coverslips were mounted with an anti-fade mounting medium and observed under a fluorescence microscope.

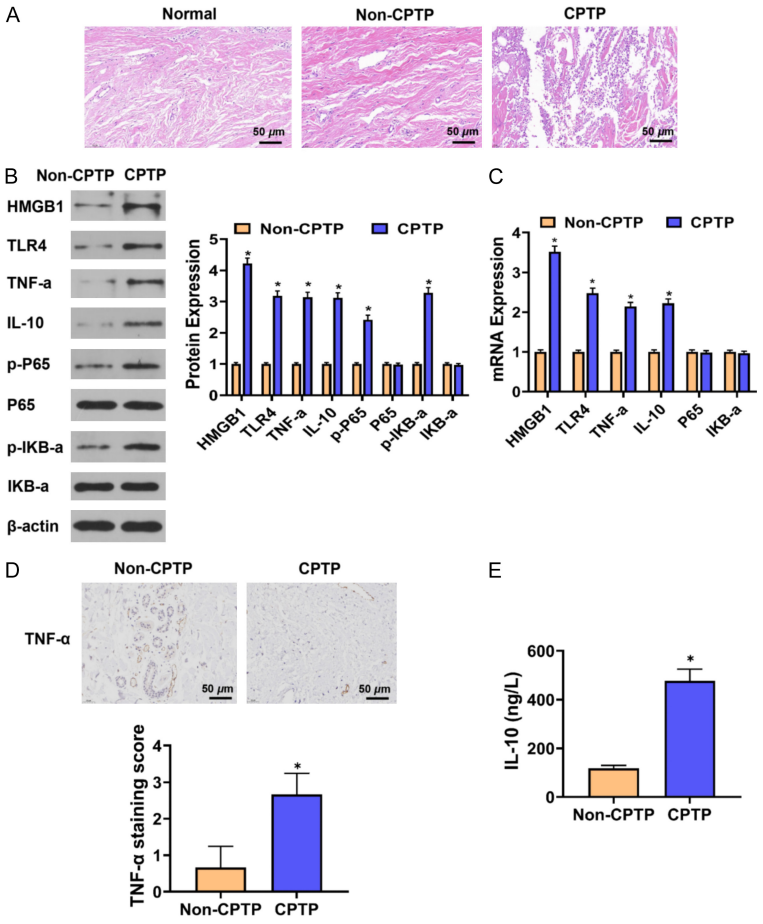

Figure 2. Differential expression of HMGB1 in CPTP. A. H&E staining of post-thoracotomy scar tissues (scale bar = 50 μm); B, C. Protein and mRNA expression of pro-inflammatory mediators and signaling molecules; D. Immunohistochemistry (IHC) showing the expression of TNF-α in scar tissues (scale bar = 50 μm); E. Enzyme-linked immunosorbent assay (ELISA) showing the expression of IL-10 in scar tissues. Note: HMGB1, High mobility group box 1; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α. *P < 0.05, compared to the non-CPTP group.

ELISA

The cultured cells were collected, and concentrations of IL-10 and TNF-α levels in cell supernatants were quantified using commercially available ELISA kits (CIOBO BIO) according to the manufacturer's instructions.

CCK-8 assay

In brief, microglial cells at logarithmic growth phase were inoculated into 96-well plates at density of approximately 2,000 cells per well and incubated at 37°C for 24 h. Then, the culture medium was discarded, and a mixture of 100 μL of fresh medium and 10 μL of CCK-8 reagent was added to each well. The cells were incubated at 37°C for 2 h. Absorbance (OD) was detected at 450 nm using a microplate reader.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software, and data were expressed as mean ± standard deviation (SD). Comparisons between two groups were conducted using the independent samples t-test; and comparisons among multiple groups were conducted using one-way ANOVA, followed with Bonferroni post hoc test. A P value < 0.05 was considered significant.

Results

Differential expression of HMGB1 in CPTP

Clinical samples from patients with and without CPTP were obtained from the Affiliated Cancer Hospital of Guangzhou Medical University to investigate pathological and molecular differences. H&E staining showed thickened collagen bundles and increased fibroblasts in the scar tissues. Compared to the scar tissues from non-CPTP group, scar tissues of CPTP group showed markedly increased inflammatory cell infiltration. In contrast, cells in the normal tissues adjacent to the scar tissue showed orderly cellular arrangement without fibroblast proliferation or inflammatory cell infiltration (**Figure 2A**). Moreover, qPCR and western blot results showed significantly increased expression of HMGB1, TLR4, IL-10, and TNF-α in the CPTP group. Additionally, the phosphorylation levels of NF-κB pathway-related proteins p65 and IκB-α were markedly increased in CPTP tissues compared to non-CPTP tissues (**Figure 2B**, **2C**). IHC and ELISA assays further confirmed a higher number of TNF-α-positive cells and increased IL-10 levels in the CPTP group (P < 0.05; **Figure 2D**, **2E**). Therefore, the development of CPTP may be related to the

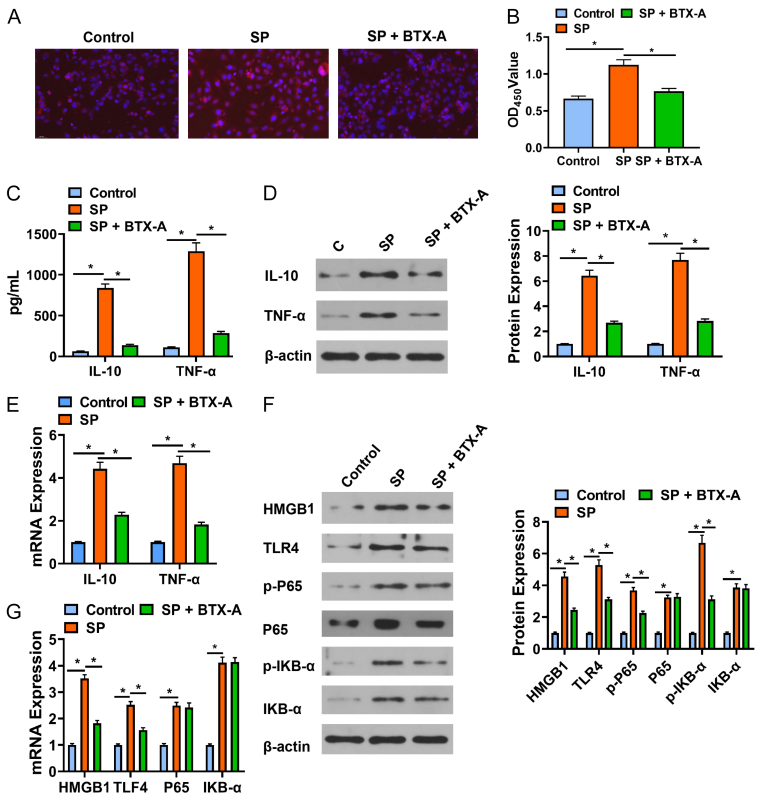

Figure 3. Effect of BTX-A on microglial function. (A) Immunofluorescence staining showing OX-42 expression; (B) Microglial cell proliferation detected by CCK-8 assay; (C) TNF-α and IL-10 levels in microglia detected by ELISA; (D, E) TNF-α and IL-10 expression in microglial cells determined by Western blot (D) and qPCR (E); (F) Protein expression of HMGB1, TLR4, P65, p-P65, IKB-α and p-IKB-α detected by Western blotting; (G) mRNA expression of HMGB1, TLR4, P65 and IKB-α detected by qRT-PCR. Note: BTX-A, botulinum toxin type; A *$P < 0.05$, compared to the SP group.

abnormal expression of HMGB1 and enhanced secretion of inflammatory factors.

BTX-A inhibited microglial inflammatory response and cellular activity

Microglial activation is considered a critical contributor to CPTP development. To investigate the effect of BTX-A on microglial activation, SP was used to simulate microglial activation in the CPTP cell model. IF staining showed that OX-42 expression was significantly increased in SP-treated cells, accompanied by elevated cell viability. In contrast, treatment with BTX-A markedly reduced OX-42 expression and OD values in SP-treated microglia ($P < 0.05$; **Figure 3A**, **3B**). ELISA results demonstrated that SP stimulation significantly increased the contents of TNF-α and IL-10 in microglial cells, whereas BTX-A treatment greatly reduced their expression (all $P < 0.05$; **Figure 3C**). These findings were further corroborated by western blot and qPCR (all $P < 0.05$; **Figure 3D**, **3E**). Further analysis revealed increased expression of HMGB1, TLR4, P65, and IκB-α and increased phosphorylation levels of P65 and IκB-α in the SP group. However, BTX-A administration notably decreased the expression of HMGB1 and TLR4 and inhibited the phosphorylation of P65 and IκB-α (**Figure 3F**, **3G**). These findings suggest that BTX-A may regulate HMGB1 expression and NF-κB pathways.

Increased HMGB1 expression enhanced microglial activity

To explore the regulatory role of HMGB1 in CPTP, HMGB1 expression was experimentally modulated, and its effects on microglia activity were assessed. Western blot and qPCR results showed that Sh-HMGB1-3 achieved the highest silencing efficiency, whereas OV-HMGB1 effectively increased both mRNA and protein levels of HMGB1 in microglial cells (**Figure 4A**, **4B**). Further studies revealed that HMGB1 overexpression significantly increased OX-42 expression and cell proliferation, whereas HMGB1 downregulation markedly reduced OX-42 expression and cell proliferation ($P < 0.05$; **Figure 4C**, **4D**). Consistently, HMGB1 overexpression significantly elevated TNF-α and IL-10 levels in microglial cells, and its inhibition attenuated the cellular inflammatory responses (**Figure 4E**, **4F**). Moreover, HMGB1 overexpression significantly increased TLR4 expression and enhanced phosphorylation levels of P65 and IκB-α, indicating that HMGB1 can positively regulate the TLR4/NF-κB pathway (**Figure 4G**) to promote microglial activation.

Activation of the TLR4/NF-κB pathway enhanced microglial activity

To clarify the role of the TLR4/NF-κB pathway in regulating microglial activation, CPTP model cells were treated with a TLR4 pathway inhibitor (TCI) or activator (MLA). The SP + TCI group

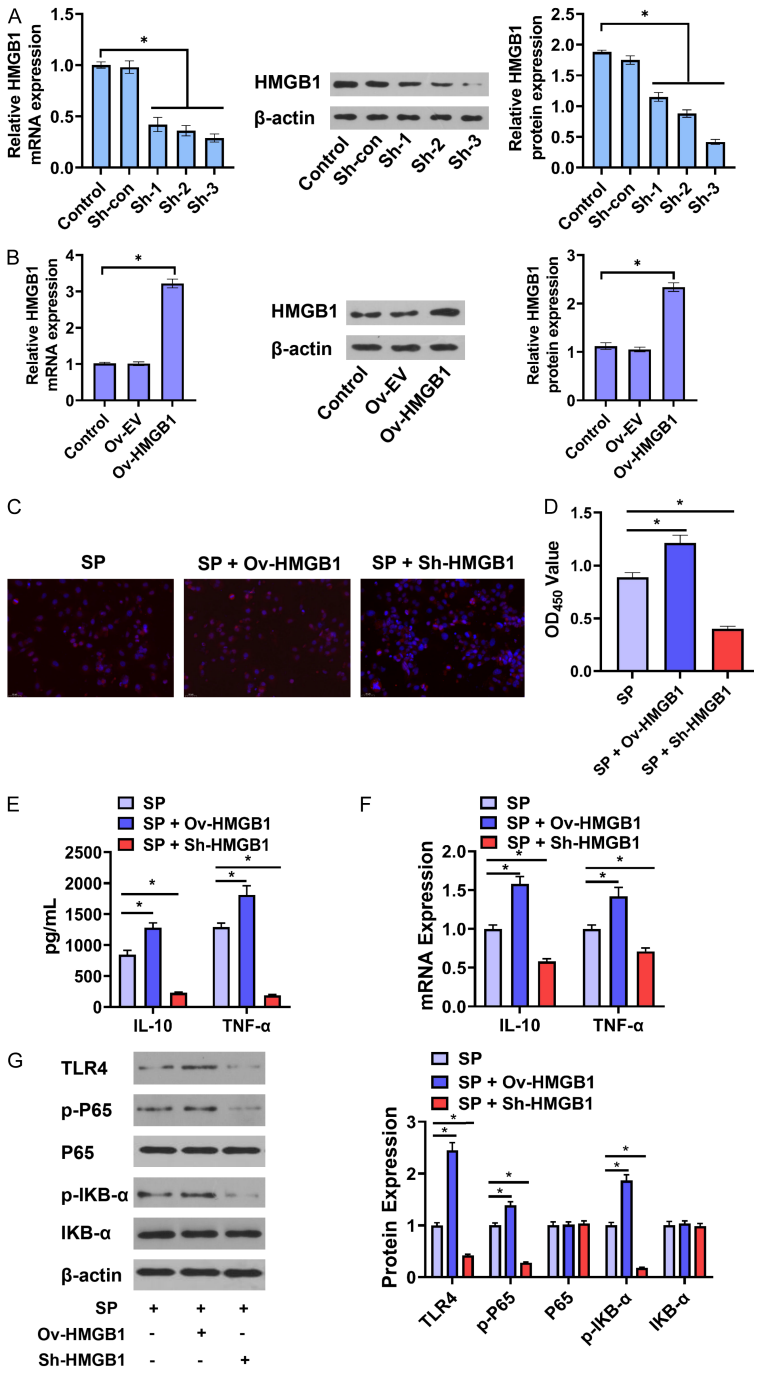

Figure 4. Effects of HMGB1 expression on microglial function. (A, B) Protein and mRNA expression of HMGB1 after transfection with Sh-HMGB1-1/2/3 (A) or Ov-HMGB1 (B); (C) OX-42 protein expression detected by immunofluorescence staining; (D) Microglial cell proliferation detected by CCK-8; (E, F) TNF-α and IL-10 expression levels measured by ELISA (E) and qRT-PCR (F); (G) TLR4/NF-κB pathway-related protein expression in microglial cells detected by western blotting. Notes: *$P < 0.05$, compared to the SP group.

exhibited decreased OX-42 expression, cell activity, and proliferation compared with the SP group. Conversely, the SP + MLA group showed increased OX-42 expression, cell activity, and proliferation (**Figure 5A**, **5B**). TLR4 activation significantly increased TNF-α and IL-10 levels in the cells, whereas inhibition of TLR4 markedly reduced the secretion of TNF-α and IL-10 in microglial cells (**Figure 5C**, **5D**). Collectively, these results suggest that HMGB1 promotes microglial activation and inflammatory response by regulating the TLR4/NF-κB pathway.

BTX-A inhibited microglial activity and alleviated CPTP through the HMGB1-mediated TLR4/NF-κB signaling pathway

Cells were further co-treated with BTX-A and Ov-HMGB1. The results showed that BTX-A administration significantly inhibited OX-42 expression and microglial activity, whereas Ov-HMGB1 transfection significantly enhanced cell activity and partially reversed the inhibitory effects of BTX-A (**Figure 6A**, **6B**). Compared to the SP + BTX-A group, Ov-HMGB1 transfection significantly increased IL-10 and TNF-α levels in microglial cells and promoted the inflammatory response (**Figure 6C**). Consistently, protein expression levels of HMGB1, TLR4, p-P65, and p-IκB-α also increased (**Figure 6D**). These findings indicate that HMGB1 upregulation attenuates the inhibitory effects of BTX-A on microglial activation, and that BTX-A alleviates CPTP by suppressing the HMGB1-mediated TLR4/NF-κB signaling pathway.

Discussion

In this study, we speculated that chronic pain following thoracotomy was related to postoperative wound-related inflammatory responses. To test this hypothesis, postoperative wound tissues from patients undergoing thoracotomy were collect-

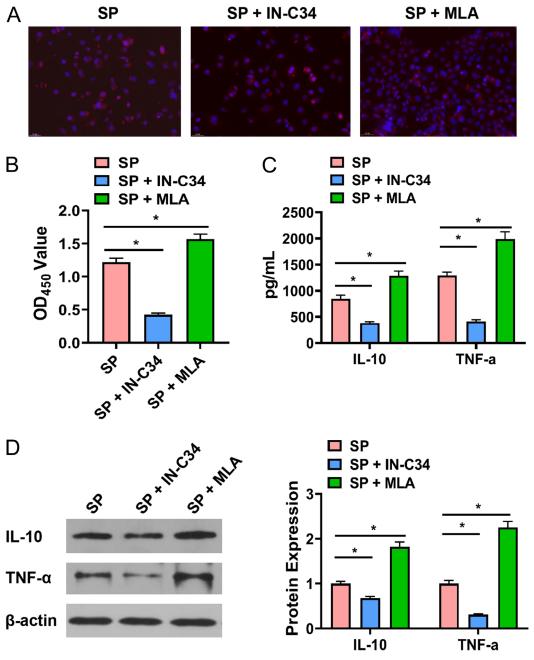

Figure 5. Effect of the TLR4/NF-κB pathway on microglial function. Microglial cells were cultured with a TLR4 inhibitor (TCI) or activator (MLA). (A) OX-42 protein expression detected by immunofluorescence staining; (B) Microglial cell proliferation detected by CCK-8; (C) TNF-α and IL-10 expression levels measured by ELISA (C) and western blotting (D). Notes: $*P < 0.05$, compared to the SP group.

ed for H&E staining. The results showed pronounced inflammatory infiltration in tissues from patients with chronic postoperative pain, suggesting that inflammation may play a critical role in the development of CPTP and warranting further mechanistic investigation.

HMGB1, a recognized pro-inflammatory mediator, plays an important role in neuritis and has been implicated in the occurrence and development of chronic pain [25]. HMGB1 can form inflammatory complexes with IL-1β and lipopolysaccharide, which subsequently activate IL-1R, TLR9, TLR2, and TLR4 to amplify the inflammatory cascades [26]. In a mouse arthritis model induced by collagen antibodies, intrathecal injection of anti-HMGB1 neutralizing antibodies was shown to reverse collagen-induced mechanical hyperalgesia [27]. Another study reported that continuous dexmedetomidine infusion reduced chronic pain scores and downregulated TLR4 and NF-κB expression in patients undergoing open-heart surgery [28]. In this study, our results demonstrated significantly elevated expression levels of HMGB1 and its associated inflammatory mediators, including TLR4, IL-10, and TNF-α in the CPTP group. Moreover, the expression of p-NF-κB (p-p65) and p-IκB-α was markedly increased in the CPTP group. These observations indicate that the occurrence of postoperative chronic pain is related to sustained inflammatory responses and may be regulated by HMGB1-mediated TLR4/NF-κB signaling pathway.

Substance P (SP) is an endogenous neuropeptide widely distributed in the central nervous system and can be expressed by multiple cell types. In clinical research, SP is often used to induce spinal microglial activation and has been widely applied in studies of neuroinflammatory responses [29]. Therefore, in this study, SP-induced microglial activation model was established to simulate cellular chronic pain and further investigate the regulatory effects of BTX-A on neuroinflammation. Our results showed that SP stimulation markedly increased OX-42 expression and enhanced cell proliferation activity; whereas BTX-A treatment significantly inhibited SP-induced OX-42 protein expression and excessive cell proliferation. OX-42 is a well-established marker of microglial activation, and detection of its expression level can be used to evaluate microglial location and activation [30, 31]. Therefore, the observed reduction in OX-42 expression suggests that BTX-A effectively attenuates microglial overactivation and may consequently alleviate pain-related inflammatory responses. Consistent with our findings, Li et al. [32] reported that BTX-A injection reversed microglial activation induced by reserpine and alleviated the microglia-mediated inflammatory response. Furthermore, combined administration of BTX-A and morphine has been shown to counteract abnormal pain-induced activation of astrocyte and microglia, enhance the analgesic efficacy of morphine, and reduce morphine-induced drug addiction [33]. These studies provide compelling evidence that BTX-A can inhibit neuroinflammation and possesses a potential neural analgesia effect.

Based on the findings from clinical data analyses, this study further elucidated the mechanisms underlying the analgesic effects of BTX-A. Analysis of inflammatory factors showed that SP induction significantly increased the expression of TNF-α, IL-10, HMGB1, TLR4, p-P65, and p-IκB-α in microglial cells. Notably, BTX-A treatment effectively counteracted SP-induced in-

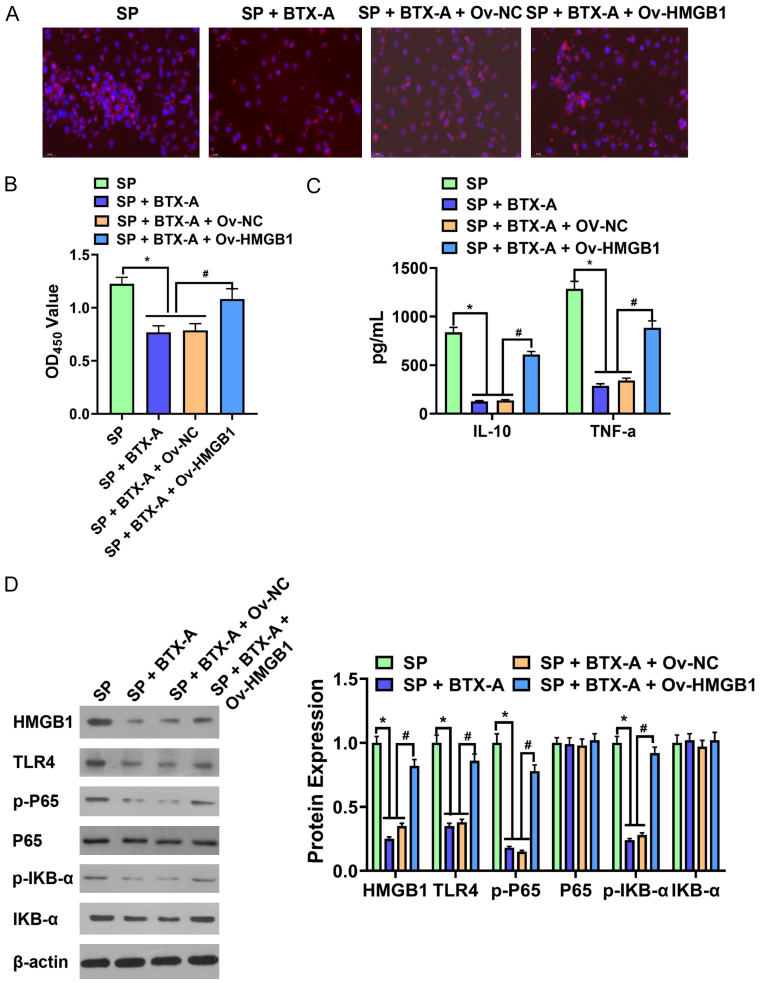

Figure 6. Mechanistic study of BTX-A-mediated regulation of CPTP cell function. CPTP cells were cultured with BTX-A and transfected with Ov-HMGB1. A. OX-42 protein expression detected by immunofluorescence staining; B. Microglial cell proliferation detected by CCK-8; C. TNF-α and IL-10 expression levels detected by ELISA; D. Protein expression levels of HMGB1, TLR4, p-P65, P65, p-IκB-α, and IκB-α determined by western blotting. Notes: $^{*}P < 0.05$, compared to the SP group; $^{\#}P < 0.05$, compared with the SP + BTX-A + Ov-HMGB1 group.

flammatory activation by suppressing the expression of these mediators and inhibiting TLR4/NF-κB pathway activation. Previous *in vitro* studies had shown that BTX-A alleviates neuropathic pain by inhibiting TLR2/MyD88 signaling and reducing the secretion of pro-inflammatory cytokines from activated microglia [34]. Additionally, BTX-A has been reported to exert anti-inflammatory effects by attenuating the phosphorylation of NF-κB, p38, and ERK1/2 in microglial cells [35]. These results indicate that microglial activation mediated by inflammatory mediators and pattern recognition receptors, such as *HMGB1*, *TLR4*, and *TLR2*, is a critical contributor to postoperative neuropathic pain. BTX-A reduces the secretion of inflammatory factors in microglia and inhibits microglial activation by inhibiting the expression of HMGB1 and TLR4/NF-κB, thereby exerting an analgesic effect.

To further determine whether the therapeutic effects of BTX-A are mediated through the HMGB1-TLR4-NF-κB pathway, an HMGB1 overexpression vector was constructed and transfected into SP-induced microglial cells, and microglial activity and related pathway gene expression were assessed. HMGB1 overexpression notably enhanced microglial activity, increased TNF-α and IL-10 expression, and amplified TLR4/NF-κB pathway activation on the basis of SP induction. In contrast, HMGB1 downregulation significantly attenuated SP-induced microglial activation and reduced cellular inflammatory responses. These findings indicate that HMGB1 expression is positively correlated with microglia activity and can positively regulate TLR4/NF-κB pathway. Studies by Xu et al. [36] also confirmed that inhibiting the HMGB1/TLR4/NF-κB signaling pathway effectively reduced excessive activation of microglia and neuroinflammation, consistent with our findings. Furthermore, in LPS-induced BV2 microglia, transfection with an Ad-HMGB1 recombinant adenoviral vector reversed the anti-inflammatory effects of glycyrrhizin and restored the activation of TLR4-NF-κB pathway [37]. Therefore, HMGB1-mediated activation of the TLR4/NF-κB pathway and secretion of inflammatory cytokines play a pivotal role in excessive activation of microglia.

Subsequently, SP-induced cells were treated with inhibitors and activators of the TLR4/NF-κB pathway, which once again verified the regulatory role of the TLR4/NF-κB pathway-

mediated inflammatory signaling in excessive microglial activation. From this, we speculate that in SP-induced cells, elevated HMGB1 expression promotes the secretion of inflammatory factors by activating the TLR4/NF-κB pathway, thereby promoting an overactivation of microglia. Finally, SP-induced cells were treated with BTX-A and transfected with Ov-HMGB1, respectively. The results showed that increased HMGB1 expression reversed the inhibitory effect of BTX-A on activated microglia, indicating that BTX-A reduces the secretion of inflammatory factors by inhibiting HMGB1-mediated TLR4/NF-κB signaling pathway, thereby preventing excessive microglial activation and exerting its analgesic effect.

Conclusion

Postoperative inflammation-mediated activation of microglia plays a critical role in the development of CPTP. BTX-A effectively reduces the secretion of inflammatory cytokines and inhibits microglial overactivation by suppressing the HMGB1-mediated TLR4/NF-κB pathway signaling, thereby alleviating chronic pain following thoracotomy. The analgesic effect of BTX-A provides a new therapeutic option for patients with CPTP. However, current evidence regarding the analgesic application of BTX-A in postoperative pain management remains limited. Further large-scale and well-designed clinical investigations are warranted to confirm the safety, efficacy, and translational potential of BTX-A for the treatment of CPTP.

Disclosure of conflict of interest

None.

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