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Thymulin treatment attenuates inflammatory pain by modulating spinal cellular and molecular signaling pathways

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ABSTRACT

Thymulin is a peptide hormone which is mainly produced by thymic epithelial cells and it has immune-modulatory and anti-inflammatory effects. In this study, we investigated the effects of different doses and various timings of thymulin intraperitoneal administration on spinal microglial activity and intracellular pathways in an inflammatory rat model of Complete Freund's adjuvant (CFA). Thymulin treatment was implemented following CFA-induced inflammation for 21 days. After conducting behavioral tests (edema and hyperalgesia), the cellular and molecular aspects were examined to detect the thymulin effect on inflammatory factors and microglial activity. We demonstrated that thymulin treatment notably reduced thermal hyperalgesia and paw edema induced by CFA. Furthermore, molecular investigations showed that thymulin reduced CFA-induced activation of microglia cells, phosphorylation of p38 MAPK and the production of spinal pro-inflammatory cytokines (TNF-α, IL-6) during the study. Our results suggest that thymulin treatment attenuates CFA-induced inflammation. This effect may be mediated by inhibition of spinal microglia and production of central inflammatory mediators which seems to be associated with the ability of thymulin to reduce p38 MAPK phosphorylation. These data provide evidence of the anti-hyperalgesic effect of thymulin on inflammatory pain and characterize some of the underlying spinal mechanisms.

1. Introduction

Chronic pain conditions frequently occur as a result of inflammation and following peripheral and central sensitization. [1]. Recent studies reported that glia cells play as important roles as neurons in generation and maintenance of chronic pain [2]. Activated spinal microglia cells release various algesic substances, particularly pro-inflammatory cytokines that enhance pain transmission by acting on primary afferent neurons, spinal intrinsic neurons and spinal glia themselves [3,4]. Based on previous studies among glia cells (microglia, astrocytes, SGCs), microglia has a leading role in chronic inflammatory pain [5,6] TNF- α is primarily produced by microglia and plays an essential role in the generation of central sensitization, modulating peripheral sensitization and persistent pain [7]. IL-6 is another cytokine produced in spinal cord that has pro and anti-inflammatory effects [8]. Accumulating evidence demonstrates that P38 Mitogen-Activated Protein Kinase (MAPK) pathway activation in spinal cord contributes to the production of pro-inflammatory cytokines and generation of neuropathic and inflammatory pain. [9–11]. Also, it has been revealed that inhibition of P38 MAPK attenuates chronic inflammatory pain [12,13].

Although immune system suppression is necessary for inflammatory pain reduction, it can also cause various unwanted side effects. Targeting the intracellular pathways by some peptides is one of the most recent approaches to pain relief which can reduce the consequences of immune system suppression. Thymulin is a peptide hormone which is mainly produced by thymic epithelial cells (TEC) and plays a role in the differentiation of T cells [14]. Besides the immune-modulatory role of thymulin, several reports have emphasized on its capability of interacting directly and/or indirectly with the nervous system [15,16]. However, intracellular mechanisms and time points which this peptide may exert its neuroimmune-modulatory effects have not been shown. Considering the role of spinal microglia in neuro-inflammation and thymuline's neuro-modulatory effects, here we aimed to investigate the modulatory effects of thymulin on pain-related behaviors, spinal microglia activation and some related intracellular

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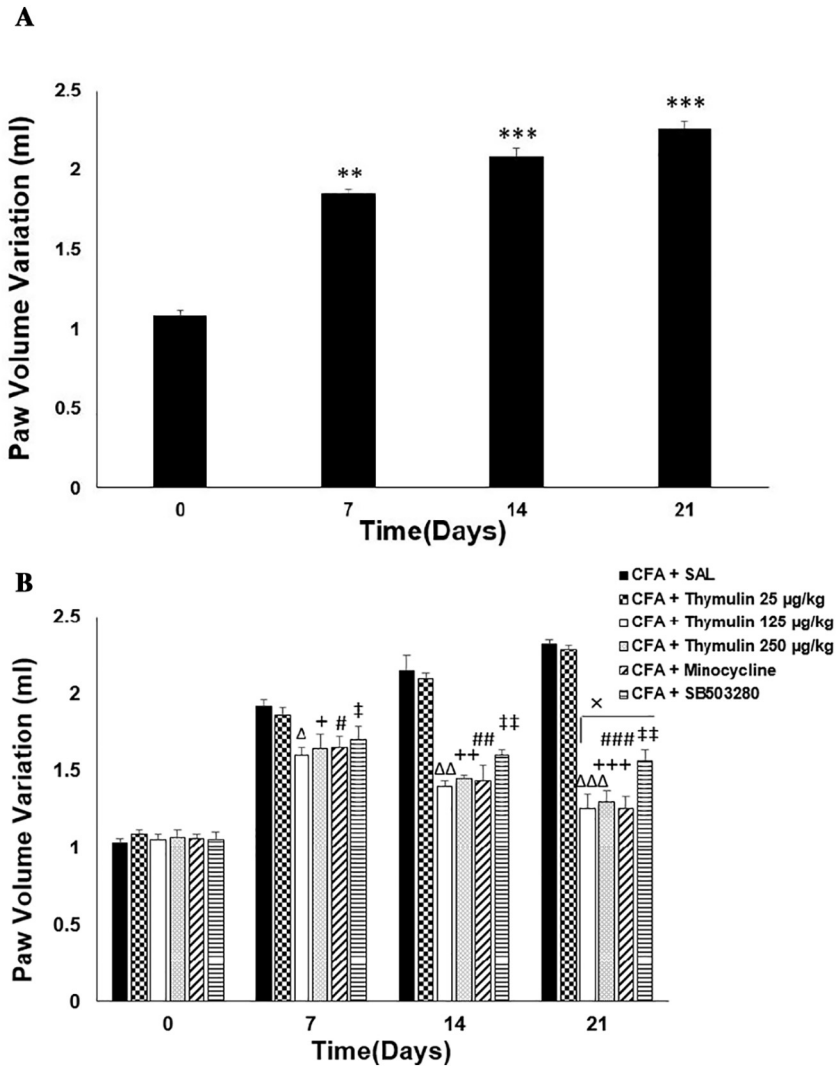

Fig. 1. Thymulin administration decreased paw volume after intra-plantar CFA injection. Results presented as Mean ± SEM (n = 6/group). A. CFA injection caused significant edema in affected paw. $^{**}p < 0.01$ and $^{***}p < 0.001$ for comparing paw volume variations between baseline and different days of the study in CFA group. B. Long-term thymulin treatment alleviated paw edema. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ and $\Delta\Delta\Delta p < 0.001$ CFA + thymulin 125 µg/kg compare to CFA + SAL. $+ p < 0.05$, $++ p < 0.01$ and $+++ p < 0.001$ CFA + thymulin 250 µg/kg compare to CFA + SAL. $\# p < 0.05$, $\#\# p < 0.01$ and $\#\#\# p < 0.001$ CFA + minocycline group compare to CFA + SAL. ‡ $p < 0.05$ and ‡‡ $p < 0.01$ CFA + SB503280 group compare to CFA + SAL. × $p < 0.05$ CFA + thymulin 125 µg/kg group compare to CFA + SB503280 group.

signaling pathways following CFA-induced acute and chronic inflammation.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (weight, 200–220 g) were used for this study. They were housed four per cage and kept under a 12 h light-dark cycle at a temperature of 23 ± 2 °C, humidity (50%–60%), and with free access to food and water. Animals were allowed to get accustomed to housing facilities for at least 1 week prior to the experiments. All experimental procedures were approved by Ethics Committee of Shahid Beheshti University of Medical Science for the use of animals in research (Code: IR.SBMU.SM.REC.1394.196), and international standards for research on experimental animals [17].

2.2. Chemicals

Complete Freund's Adjuvant (CFA), Thymulin and Minocycline, were purchased from Sigma (St. Louis, MO). The p38 MAPK inhibitor, SB203580, was purchased from Promega/USA. Western blot antibody including antibody against ionized calcium-binding adaptor molecule-1 (Iba-1) (#ab108539) was purchased from Abcam (Cambridge, UK); also beta-actin (4970), phospho-P38 (9211), total P38 antibody (9212) and secondary horseradish peroxidase (HRP-conjugated) (7074) were purchased from Cell Signaling Technology Company (Danvers, MA, USA). Immunohistochemistry reagents envision dual-link system horseradish peroxidase as a secondary antibody and 3, 3′-diaminobenzidine (DAB) was supplied from Dako (Denmark). Other reagents were also of analytic grade and were obtained from available local commercial sources.

2.3. Experimental procedures and local paw inflammation induction

Peripheral chronic inflammatory pain model was established according to our previous study [18].100 µl CFA was injected subcutaneously on day zero into the plantar surface of the right hind paw. The first day after CFA injection unilateral inflammation was established in injected hind paw. In order to determine the effect of thymulin on inflammatory pain model and the effectiveness of the treatment, a series of experiments were performed. Rats were randomly divided into different experimental groups, as follows: (a) CFA, (b) CFA control (mineral oil), (c) CFA + 0.9% saline, (d) CFA + thymulin 25 µg/kg, (e) CFA + thymulin 125 µg/kg, (f) CFA + thymulin 250 µg/kg, (g) CFA + minocycline, (h) CFA + SB203580. According to the study procedure, each group was divided into four subgroups based on different time points of the study (days 0, 7, 14, and 21; n = 6 for behavioral and molecular studies). All treatments began one day after CFA injection. Thymulin was administered by daily intraperitoneal (i.p.) injection at 25,125 and 250 µg/kg in different groups. We selected the optimal dose of thymulin for molecular studies by administration of

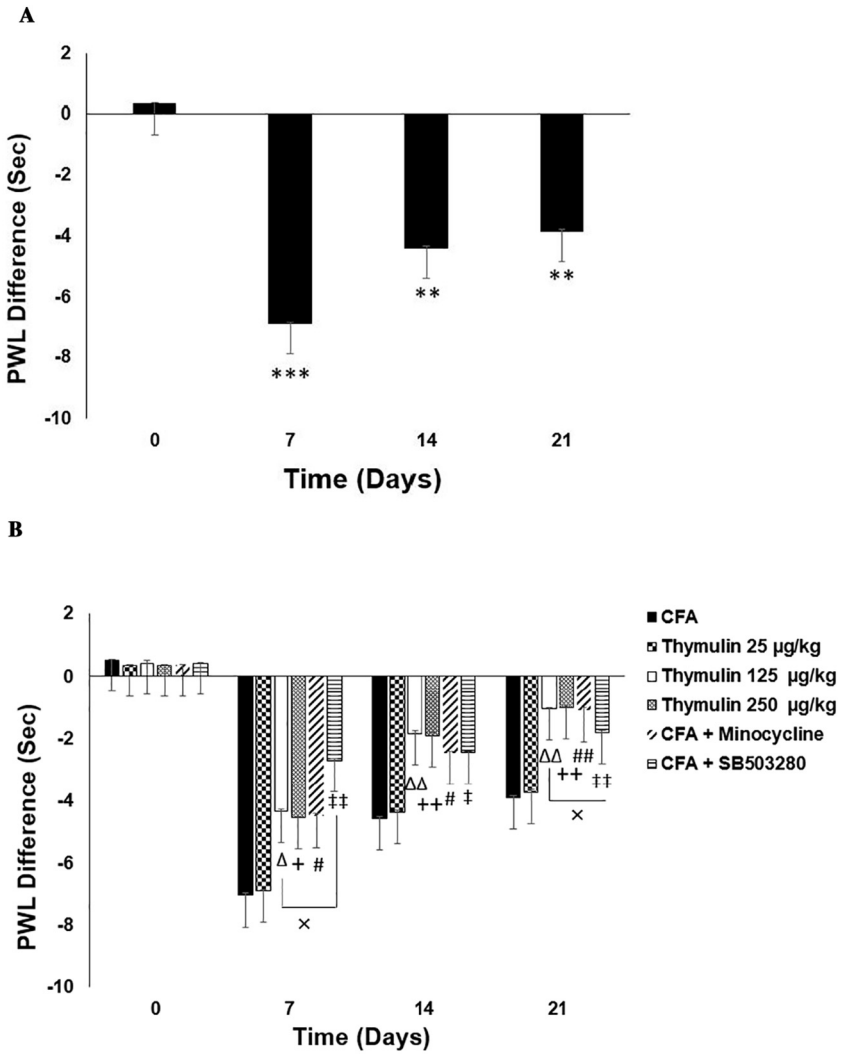

Fig. 2. PWLs variations in experimental groups at different time-points of study. Results presented as Mean ± SEM (n = 6/group). A. CFA injection into the right hind paw of rats caused significant hyperalgesia at 7th day but, hyperalgesia reduced at 14th and 21st days. ***$p < 0.001$ and **$p < 0.01$ for comparing hyperalgesia variations between baseline and different days of the study in CFA group. B. Thermal hyperalgesia induced by CFA injection was significantly attenuated by administration of thymulin. Δ $p < 0.05$ and ΔΔ $p < 0.01$ CFA + thymulin125 µg/kg compared to CFA + SAL. + $p < 0.05$ and + + $p < 0.01$ CFA + thymulin250 µg/kg compare to CFA + SAL. # $p < 0.05$ and ## $p < 0.01$ CFA + minocycline compare to CFA + SAL. ‡ $p < 0.05$ and ‡‡ $p < 0.01$ CFA + SB503280 group compare to CFA + SAL. × $p < 0.05$ CFA + thymulin 125 µg/kg group compare to CFA + SB503280 group.

different doses and evaluating the results of behavioral sections. Thymulin, minocycline hydrochloride and SB203580 were dissolved in 0.9% saline. Minocycline hydrochloride was administered by daily i.p. injection at a dose of 40 mg/kg [19]. SB203580 (p38 MAPK inhibitor) re-suspended 1 mg in 265 µl of vehicle to produce a stock solution of 10 mM; for inhibition of p38 phosphorylation, 70 nM/100 µM of that stock was administered daily via i.p. injection. [20]. At the end of each period (days 0,7,14 and 21), after conducting behavioral tests, the rats were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.), decapitated and then the lumbar spinal cord was quickly removed for the assessment of cellular and molecular variations during different stages of the study. Also, the same rats were evaluated for edema and thermal hyperalgesia.

2.4. Body weight and food intake study

Rats were weighed every week. Food was weighed, and the average daily intake was calculated.

2.5. Measurement of paw edema

Inflammation induction by CFA administration was assessed by measurements of paw volume on days 0, 7, 14 and 21 of the study by displacement of an electrolyte solution in a plethysmometer (model 7141; UGO Basile, Italy) [21]. The rat's hind paw was immersed in a chamber containing electrolyte solution up to the tibiotarsal joint. Then, the volume of liquid displacement was indicated on a digital displayer. Experiments carried out twice for each paw, and the average volume displacement was calculated. The results are expressed as the algebraic difference between the volume (ml) of the injected paw before CFA injection (day 0) and during different stages of the study.

2.6. Thermal hyperalgesia assessment

Thermal hyperalgesia was determined by measuring Paw Withdrawal Latencies (PWLs), using a radiant heat (UGO Basile, Verse, Italy). As with our previous study [20], rats were placed in a Plexiglas cage, after habituation the heat source was directed to the plantar surface of the hind paw. A digital timer automatically recorded the duration between the start of stimuli and paw withdrawal. However, a cut-off time of 25 s was set to prevent tissue damage. Withdrawal latency was averaged three times for each hind paw at 5 min interval. Then, the mean value for the inflamed paw was subtracted from that for the other paw and the result was considered as the hyperalgesia sign in the injured paw.

2.7. Spinal cord TNF-α and IL-6 level variation assessment by ELISA

Lumbar spinal cord TNF-α and IL-6 level were assessed by related tissue ELISA kits according to the manufacturer's protocols (Mybiosource, San Diego, USA). The minimum detectable dose is typically < 0.078 pg/ml and 1 pg/ml respectively for IL-6 and TNF-α.

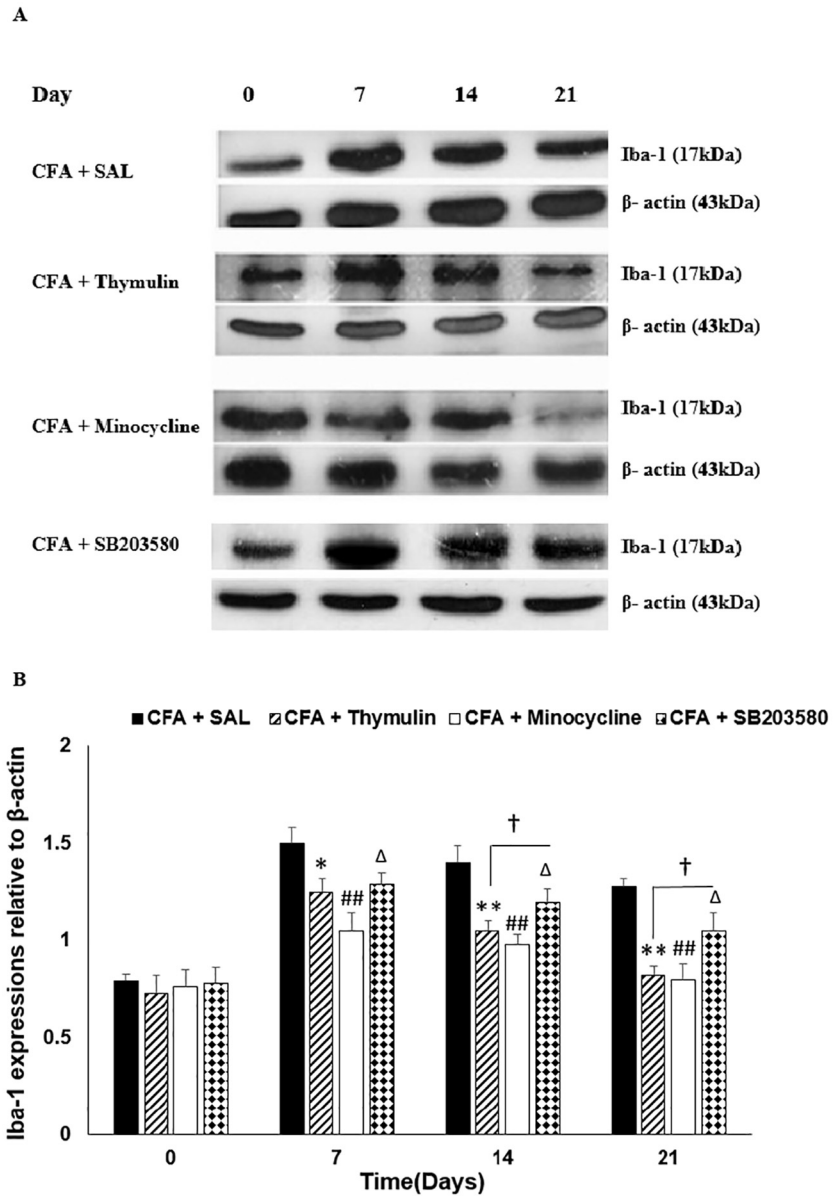

Fig. 3. A. Western blots of spinal Iba-1(17 kDa) expression after hind paw CFA-injection with β - actin (43 kDa) as the loading control. B. Results presented as Mean ± SEM (n = 6/group). All results were expressed as Iba-1/β -actin ratios. The expression of Iba-1 increased in the CFA + SAL group during the study, but thymulin treatment decreased the expression of Iba-1. *p <0.05 and **p <0.01 CFA + thymulin group compare to CFA + SAL. ## p <0.0.01 CFA + minocycline group compare to CFA + SAL. Δ p <0.05 CFA + SB203580 group compare to CFA + SAL. † p <0.05 CFA + thymulin group compare to CFA + SB203580 group.

Each measurement was repeated for three times.

2.8. Western blot analysis

Lumbar spinal cord Western blotting was performed to determine molecular mechanisms involved in the anti-inflammatory effects of thymulin. Briefly, the lumbar spinal cord of experimental rats was quickly excised, then homogenized in the cold RIPA lysis buffer including proteinase and phosphatase inhibitors. The supernatant was collected after centrifuging (13,000 × *g* for 45 min at 4 °C), and the concentration of proteins was identified by the Bradford method. Thereafter, equal amounts of protein were separated by 12% polyacrylamide gel electrophoresis and the proteins were transferred to PVDF membranes. The non-specific bands on PVDF paper were blocked with 2% Bovine Serum Albumin (BSA), then the membranes were incubated with the primary antibody (Iba-1, P38, total P38 and β-actin) overnight in 4 °C. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody for 1 h at 24 °C. Immunoreactive bands were visualized by using ECL select kit and quantified by using a densitometric analysis with Image J (NIH, USA). Each test was repeated three times for each group.

2.9. Immunohistochemistry

Rats for immunohistochemical assessment were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused with saline followed by a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate-buffered (pH: 7.4). The lumbar spinal segments were removed and post-fixed in the same fixative for 24–48 h for paraffin embedding. Tissues were sectioned at a thickness of 5 μm on a microtome with an interval of 300 μm, and then they were immersed in 10% H_2O_2/methanol for 10 min to reduce endogenous peroxidase activity. After that, the slides were washed with Tri –buffer saline (pH = 7.4) and incubated in citrate buffer (pH = 6) for 11 min in 120 °C.

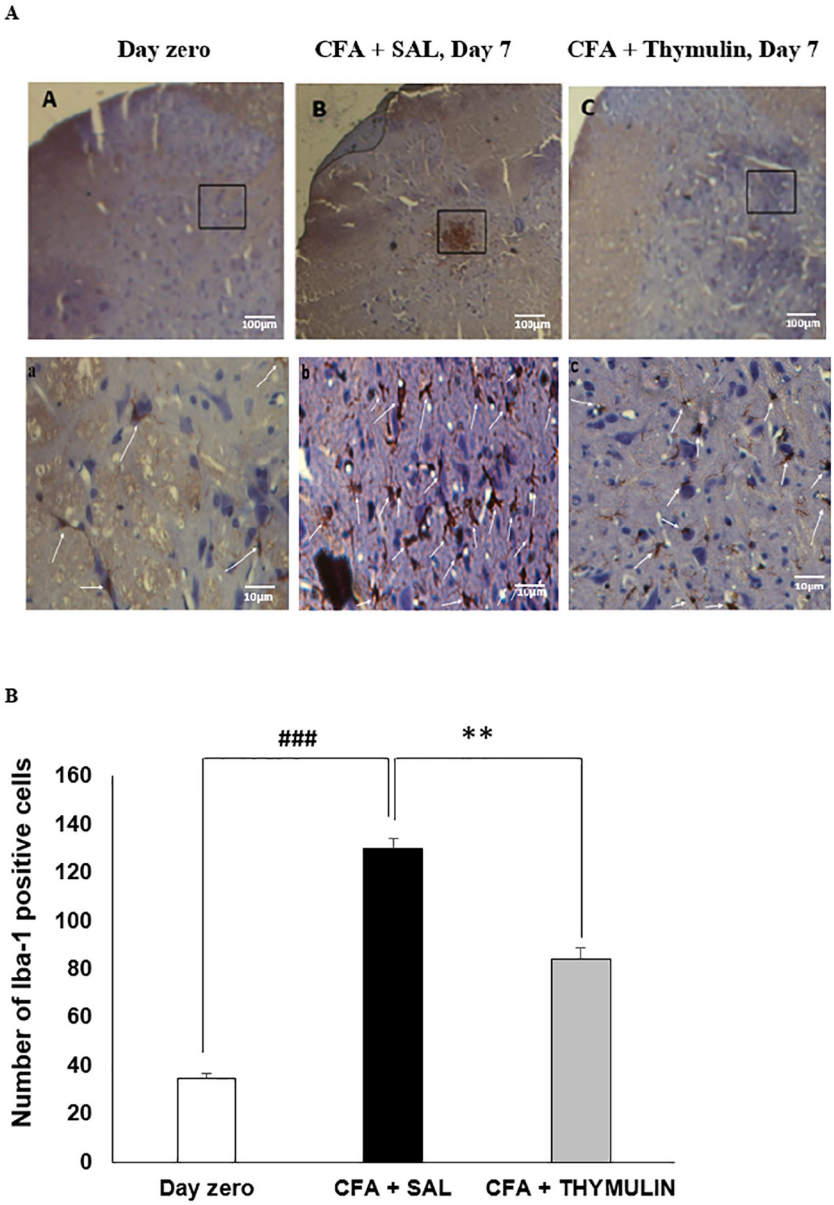

Fig. 4. A. Immunohistochemistry images of the dorsal horn of spinal cord before CFA injection, 7 days after CFA-induced inflammation and thymulin treatment, incubated with an antibody recognizing Iba-1. Images (A–C) were viewed by using a 4× and (a–c) by using a 40× lens. Scale bars are 100 µm for (A–C) and 10 µm for (a-c). B. Results presented as Mean ± SEM (n = 3/group). Diagram indicating the number of Iba-1 positive microglia cells. ### $p < 0.001$, CFA + SAL vs. Day zero group; **$p < 0.01$, CFA + thymulin vs. CFA + SAL.

Afterward, tissues were blocked in BSA2% for 10 min for antigen retrieval then incubated primary antibody for identification of Iba-1 (Rabbit monoclonal Iba-1; Abcam; 1:100) at 4 °C overnight. Slides were washed again in Tris wash buffer (pH = 7.4) and incubated in Envision Dual link System horseradish peroxidase as a secondary antibody (1:100, Dako, Denmark) for 1 h. Slides were washed again in Tris wash buffer (pH = 7.4). 3,3′-diaminobenzidine (DAB; Dako, Carpinteria, CA) reaction was used to yield a brown (DBH). After immunostaining, sections were briefly counterstained with hematoxylin for 10 min, then washed in tap water and dehydrated in ascending alcohols, cleared in xylene and covered with a coverslip. Olympus AX70 microscopes (Japan) with a DP11 digital camera (magnification of 40×) were used to take the pictures. Five random fields of different spinal cord regions were investigated. OLYSIA BioReport software (Olympus Optical Co. Ltd., Japan) was used. The number of Iba-1 positive cells in the same five squares of a grid in each field was counted and the final count was reported as number per field.

2.10. Statistical analysis

Data were presented as the mean and standard errors of the means (Mean ± SEM). Statistical comparisons were analyzed by repeated measurements for evaluation of within-groups differences and one-way analysis of variance (ANOVA), followed by Post hoc Tukey's test for multiple comparisons. P values of $0<0.05$ were accepted as statistically significant. Statistical analysis was performed using the IBM SPSS Statistics for Windows, version 22.0 (SPSS Inc., Armonk, NY).

3. Results

3.1. Body weight and food intake

We measured body weight and food intake. There were no significant differences between thymulin treated rats and normal rats in those parameters (data are not shown).

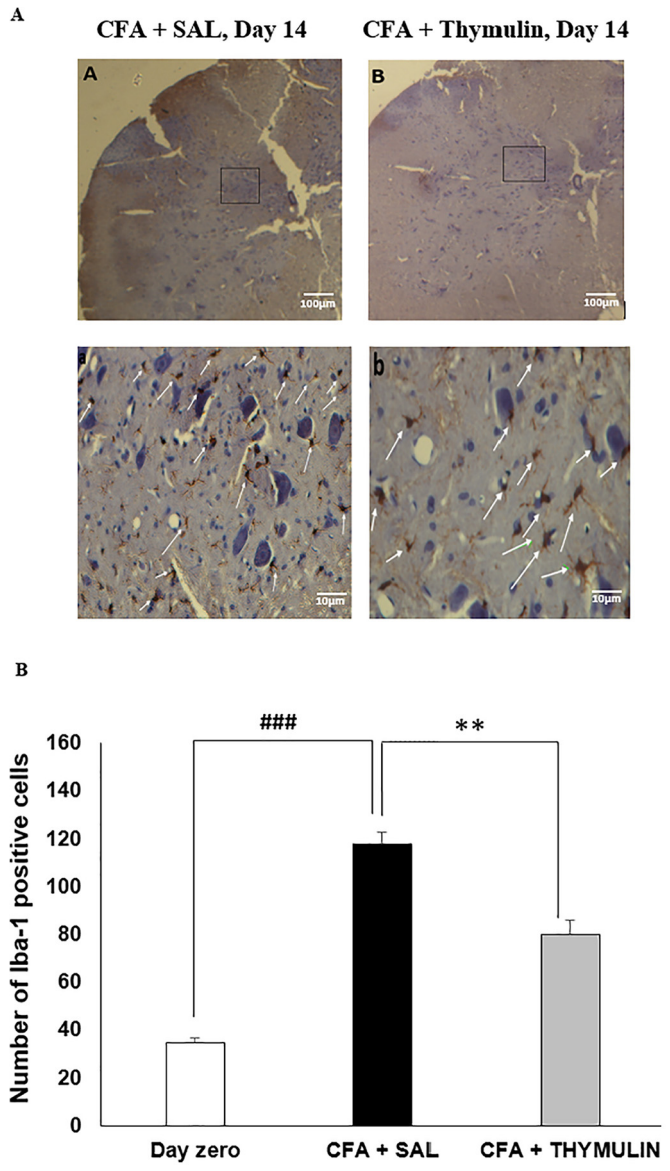

Fig. 5. A. Immunohistochemistry images of the dorsal horn of spinal cord 14 days after CFA-induced inflammation and thymulin treatment, incubated with an antibody recognizing Iba-1. Images (A and B) were viewed by using a 4× and (a and b) by using a 40× lens. Scale bars are 100 µm for (A–C) and 10 µm for (a–c). B. Results presented as Mean ± SEM (n = 3/group). Diagram indicating the number of Iba-1 positive microglia cells. ### $p < 0.001$, CFA + SAL vs. Day zero group; **$p < 0.01$, CFA + thymulin vs. CFA + SAL.

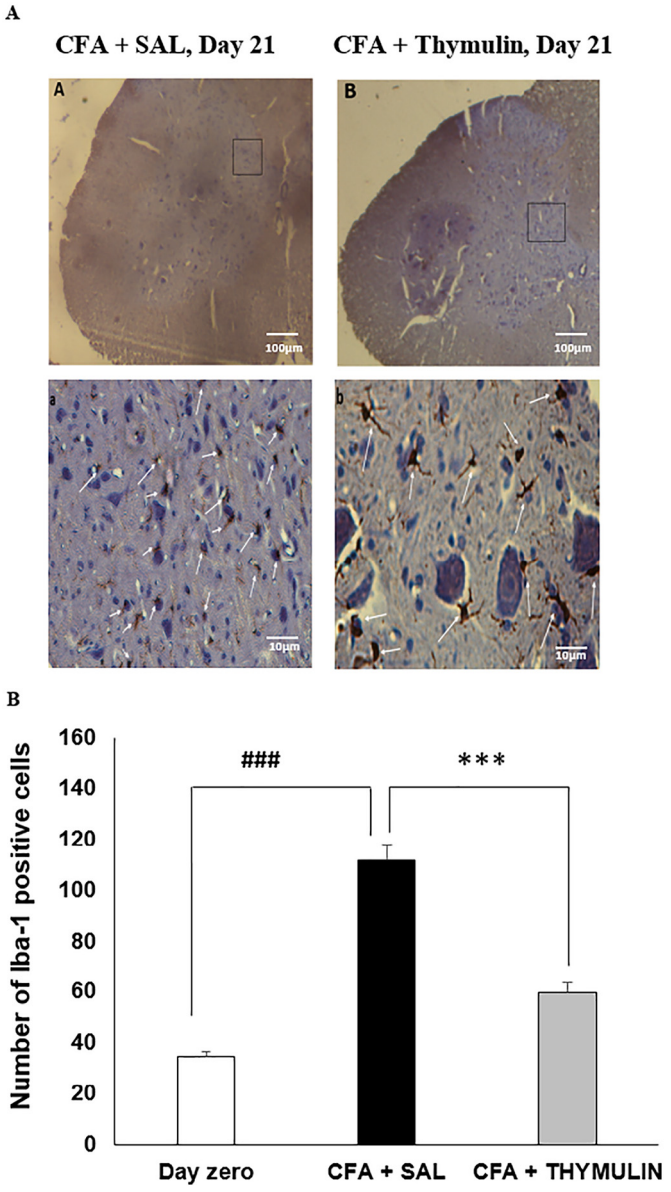

Fig. 6. A. Immunohistochemistry images of the dorsal horn of spinal cord 21 days after CFA-induced inflammation and thymulin treatment incubated with an antibody recognizing Iba-1. Images (A and B) were viewed by using a 4 x and (a and b) by using a 40× lens. Scale bars are 100 µm for (A–C) and 10 µm for (a–c). B. Results presented as Mean ± SEM (n = 3/group). Diagram indicating the number of Iba-1 positive microglia cells. ### $p < 0.001$, CFA + SAL vs. Day zero group; **$p < 0.001$, CFA + thymulin vs. CFA + SAL.

3.2. Thymulin treatment reduced CFA-induced paw edema

Unilateral CFA injection into the right hind paw caused marked inflammation and a significant increase in paw volume (edema) which was continued until the 21st day of the study ($p < 0.01$ for day 7 and $p < 0.001$ for days 14 and 21) (Fig. 1A). There were no significant differences in paw volume in the CFA control group following injection of sterile mineral oil on different days of study (Hence, the results of the CFA control group were not shown graphically).

Daily (i.p.) administration of thymulin by 25 µg/kg dose did not change the paw volume compared to the control group during different days of study (Fig. 1B). However, thymulin treatment by (125 and 250 µg/kg) after CFA injection significantly reduced paw volume relative to CFA + SAL group ($p < 0.05$ for day 7 and $p < 0.01$ for day 14 and $p < 0.001$ for day 21) (Fig. 1B). Since there was no significant difference between 125 and 250 µg/kg doses on paw volume variation; so, we selected 125 µg/kg doses as an optimally effective dose. Results demonstrated that edema reduction by thymulin administration (125 µg/kg) in CFA + Thymulin group on day 21 is more than days 7 and 14 (Fig. 1B). There were no significant differences in paw volumes between CFA and CFA + SAL (vehicle) groups, then CFA + SAL group was selected as a control group for comparing with other groups. There was significant difference in paw edema between CFA + thymulin (125 µg/kg) treated and CFA + SB 203580 groups on day 21 of study ($p < 0.05$) (Fig. 1B). Furthermore, no significant differences have been observed between thymulin and minocycline (as a positive control group) treated groups after CFA injection during different days of study (Fig. 1B).

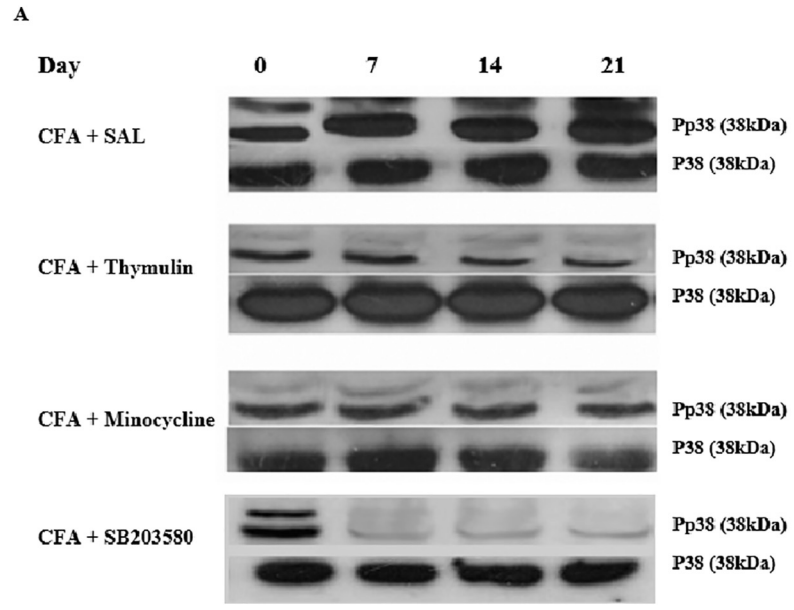

Fig. 7. A. Western blots of spinal P-p38MAPK (38 kDa) expression after CFA intraplantar injection with p38MAPK as the loading control in different experimental groups. B. Western blot analysis showed that thymulin treatment reduced P-p38MAPK / p38MAPK ratio follow CFA-induced inflammation. Results presented as Mean ± SEM (n = 6/group). All results were expressed as P-p38MAPK/p38MAPK ratios. *$p < 0.05$ and ***$p < 0.001$ CFA + thymulin group compare to CFA + SAL group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ CFA + minocycline group compare to CFA + SAL group. ΔΔΔ $p < 0.001$ CFA + SB203580 group compare to CFA + SAL group. ††† $p < 0.001$ CFA + thymulin group compare to CFA + SB203580 group.

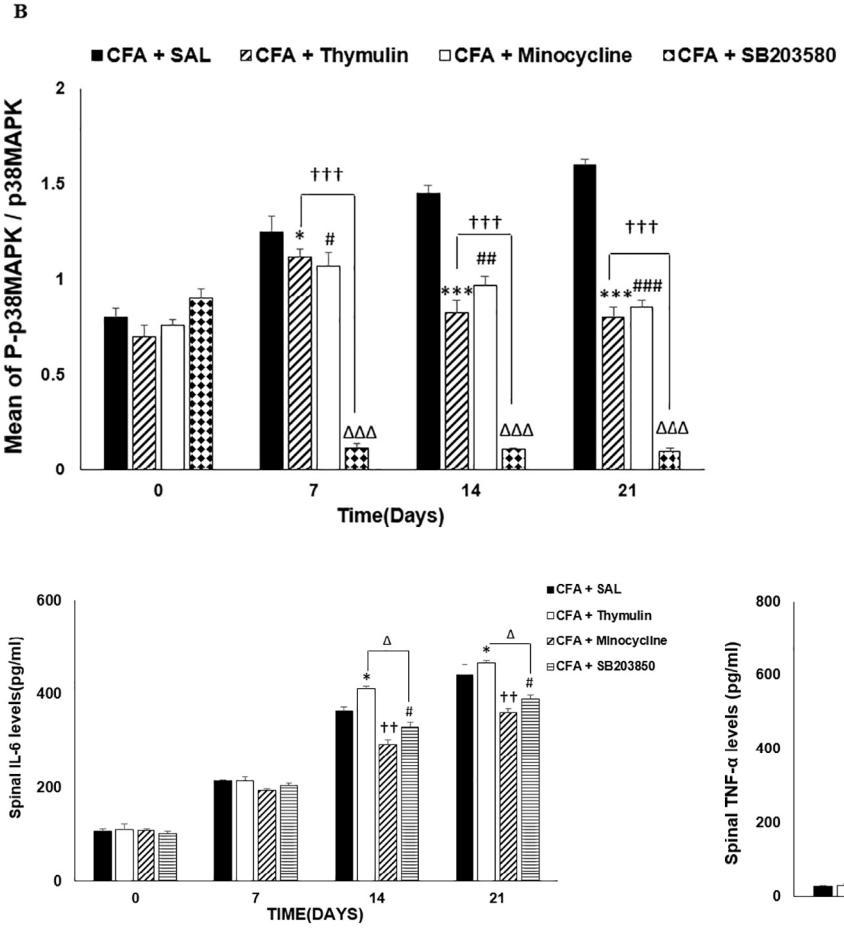

Fig. 8. ELISA analysis of spinal IL-6 level during different stages of CFA-induced inflammation. Results presented as Mean ± SEM (n = 6/group). *$p < 0.05$ CFA + thymulin group compare to CFA + SAL group. †† $p < 0.01$ CFA + minocycline group compare to CFA + SAL group.# $p < 0.05$ CFA + SB203850 group compare to CFA + SAL group. Δ $p < 0.05$ CFA + thymulin group compare to CFA + SB203850 group.

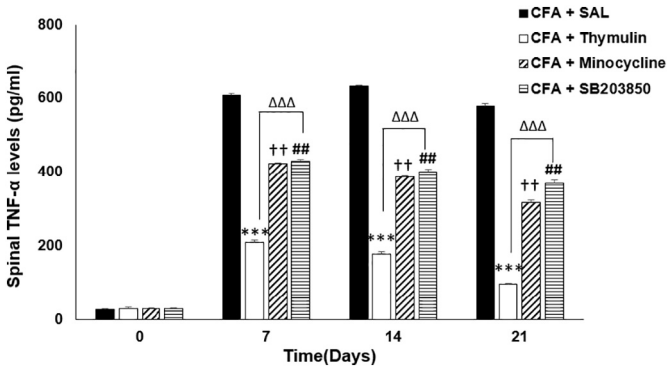

Fig. 9. ELISA analysis of spinal TNF-α level during different stages of CFA-induced inflammation. Results presented as Mean ± SEM (n = 6/group). ***$p < 0.001$ CFA + thymulin group compare to CFA + SAL group. †† $p < 0.01$ and ††† $p < 0.001$ CFA + minocycline group compare to CFA + SAL group. ## $p < 0.01$ CFA + SB203850 group compare to CFA + SAL group. ΔΔΔ $p < 0.001$ CFA + thymulin group compare to CFA + SB203850 group.

3.3. Thymulin treatment reduced CFA-induced thermal hyperalgesia

We observed variations of thermal hyperalgesia in the inflamed paw during the study by measuring thermal paw withdrawal latency (PWLs). Hyperalgesia increased at 7th day following CFA injection and gradually decreased at 21 days ($p < 0.001$ for day 7 and $p < 0.01$ for days 14 and 21) (Fig. 2A). Intraplantar injection of sterile mineral oil to the rat's right hind paw caused no significant variations in hyperalgesia during the study in the control group (Hence, the results of the control group were not shown graphically).

Daily (i.p.) administration of thymulin by 25 μg/kg dose had no significant effects on the reduction of hyperalgesia during different days of study in comparison to the control group. However, thymulin administration by 125 and 250 μg/kg doses in CFA + thymulin groups significantly reduced heat hyperalgesia in comparison to CFA + SAL group ($p < 0.05$ for days 7 and 14 and $p < 0.01$ for day 21) (Fig. 2B). Considering these behavioral results and due to the fact that thymulin 125 μg/kg dose had a subtractive effect on thermal hyperalgesia induced by CFA injection same as thymulin 250 μg/kg (Fig. 2B), so 125 μg/kg dose of thymulin is chosen as an optimal dose. Hyperalgesia variations between CFA + SAL and CFA groups were not indicated a significant difference between those groups during different time points of study, then CFA + SAL group was considered as a control group for more between groups assessments. There was significant difference in paw withdrawal latency between CFA + thymulin (125 μg/kg) treated and CFA + SB 203580 groups on days 7 and 21 ($p < 0.05$ for days 7 and 21) (Fig. 2B).

3.4. Thymulin administration reduced spinal Iba-1 expression after CFA stimulation

Our data showed that, intraplantar injection of CFA can activate microglia in the lumbar spinal cord since, spinal microglial marker, Iba-1 protein expression observable increased on days 7, 14, and 21 compared to day zero of this study ($p < 0.001$ for days 7 and 14 and $p < 0.01$ for day 21) (Fig. 3A and B). Treatment with an effective dose of thymulin (regarding behavioral results) changed microglial activity follow CFA-induced inflammation. As shown in Fig. 3A and B, in the CFA + thymulin group, expression of spinal Iba-1 was significantly decreased till 21 days of study in comparison to CFA + SAL group ($p < 0.05$ for day 7 and $p < 0.01$ for days 14 and 21). (Fig. 3A and B). Spinal Iba-1 expression in CFA + thymulin(125 μg/kg) treated group indicated significant difference with CFA + SB 203580 group on days 14 and 21 ($p < 0.05$ for both days 7 and 21) (Fig. 3B).

For further investigation of microglia activation, we examined Iba-1 expression in the lumbar spinal cord by immunohistochemistry staining at different time points of the study. On day zero (before CFA injection), a few Iba-1 positive microglia cells were expressed (Fig. 4A and B). In CFA + SAL group on the 7th day of study, a large number of Iba-1 positive microglia cells demonstrated severe immunoreactivity and the microglial processes were shortened and thickened in comparison to day zero group ($p < 0.001$) (Fig. 4A and B). Results revealed that thymulin treatment significantly reduced the number of Iba-1 positive microglia cells in the dorsal horn of the spinal cord after 7 days following CFA-induced inflammation ($p < 0.01$) (Fig. 4A and B). Our results showed that there was a significant increase in a number of Iba-1 positive microglia cells at 14 and 21 days after CFA injection in CFA + SAL groups in comparison to day zero group ($p < 0.001$) (Figs. 5 and 6). Thymulin treatment after 14 and 21 days markedly decremented number of Iba-1 positive microglia cells compare to CFA + SAL group ($p < 0.01$ for day 14 and $p < 0.001$ for day 21) (Figs. 5A and 6B), so that at day 21 of the experiment, no significant distinction was perceived in number of Iba-1 positive cells in CFA + Thymulin group compared to CFA + SAL group (Fig. 6A and B).

3.5. Thymulin treatment reduced spinal p38 MAPK phosphorylation after CFA stimulation

We performed Western blot to evaluate the expression of lumbar spinal P-p38MAPK/p38MAPK at different time points of study (Fig. 7A). Our data stated that the level of P-p38MAPK/p38MAPK increased at 7, 14 and 21 days after CFA injection ($p < 0.01$ for day 7 and $p < 0.001$ for day 14 and 21) (Fig. 7B). Thymulin long-term administration in CFA + thymulin group significantly decreased the level of spinal P-p38MAPK/p38MAPK in comparison to CFA + SAL group ($p < 0.05$ for day 7 and $p < 0.001$ for days 14 and 21) (Fig. 7B). Regarding our results, phosphorylation ratio of p38 MAPK (P-p38/p38) decrement in CFA + thymulin group on days 14 and 21 of the study was significantly more than day 7 (Fig. 7B). Although SB 203580 treatment inhibited phosphorylation of p38 MAPK in CFA group, but thymulin administration in CFA group caused significant decrease in Pp38/p38 MAPK expression in spinal cord too ($p < 0.001$ for all days 7, 14 and 21) (Fig. 7B).

3.6. Thymulin treatment changed spinal cytokines level after CFA stimulation

Our findings indicated that spinal IL-6 level significantly increased after intraplantar injection of CFA during different stages of the study ($P < 0.5$ for day 7, $P < 0.01$ for day 14 and $P < 0.001$ for day21). Moreover, daily thymulin (effective dose) treatment in CFA + thymulin group significantly increased spinal IL-6 in comparison to CFA + SAL group on days 14 and 21 of study ($P < 0.05$). Spinal level of IL-6 in CFA + SB203580 group on days 14 and 21 was significantly lower than CFA + thymulin group ($P < 0.05$ for days 14 and 21) (Fig. 8).

Additionally, CFA-induced inflammation caused remarkable up-regulation on spinal TNF-α on 7th, 14th and 21st days of study ($P < 0.001$ for days 7, 14 and 21). Thymulin treatment effectively decreased CFA-induced up-regulation of spinal TNF-α. There were significant differences in spinal TNF-α level between the CFA + SAL and CFA + thymulin groups on 7th, 14th and 21st days of study ($P < 0.001$) (Fig. 9). Also, thymulin treatment in CFA injected group could reduce spinal TNF-α level more than SB203580 treatment on days 7, 14 and 21 after CFA injection (P0.001) (Fig. 9).

4. Discussion

In this study, we investigated the anti-nociceptive and anti-neuroinflammatory effects of thymulin during CFA- induced acute and chronic inflammation. Our data provide compelling evidence that, long-term treatment by thymulin attenuated CFA-induced hyperalgesia via decreasing the activation of spinal microglia and production of central pro-inflammatory cytokine (TNF-α) by inhibiting spinal intracellular p38 MAPK signaling pathways.

Inflammation is a prominent property of the CFA model, as indicated by early inflammatory cells activation [22] and cytokine production [23,24]. Studies revealed that non-neuronal cells such as immune cells and glial cells have been implicated in the pathogenesis of chronic inflammation [25]. Microglia was activated in the spinal cord following peripheral nerve injury or tissue inflammation [26]. They exhibit dynamic plasticity by switching from quiescent or resting phenotype to a reactive phenotype after an injury to the nervous system and then act as immune responsive cells to modulate neuronal activity [27]. The activated glial cells contribute to enhancement and maintenance of chronic pain by releasing neuromodulators, such as growth factors, pro-inflammatory cytokines and chemokines [28,29]. Microglia inhibition has been considered as a therapeutic target for the development of novel anti-inflammatory agents [30]. Our results are in general agreement with previous studies and demonstrate the microglial marker; Iba-1 was increased in the spinal cord after CFA injection, which was supported by immunohistochemistry staining. Sun et al.

showed that spinal microglia was activated at day 3 after peripheral CFA-induced inflammation [31], but our results indicated that spinal microglia increased activity can be continued till 21 days after CFA injection which it can suggest persistent and longtime roles for these cells during acute and chronic phase inflammation. Our current findings also expand prior works that indicated microglia activation may concern as one of the important sources of spinal pro-inflammatory cytokines such as IL-6, and TNF-α following peripheral inflammation [32]. Our results not only revealed that increased levels of TNF-α in the spinal cord (which is correlated to microglial activation) was involved in hyperalgesia variations during different phases of CFA-induced inflammation but also stated that daily thymulin (125 μg/kg) administration can reduce hyperalgesia via prevention of spinal TNF-α accumulation. Thymulin indicated inhibitory effects on acute inflammatory responses due to cytokine production during peripheral inflammation [33]. It is reported that pre-treatment with peptide analog of thymulin prevented acute hyperalgesia and reduced serum levels of pro-inflammatory cytokines in endotoxin-induced inflammation [34]. It was already demonstrated that spinal TNF-α has an important role in inducing and maintaining pain following peripheral inflammation [29]. Some studies demonstrated TNF-α plays an important role in central sensitization so that inhibition of the TNF-α signaling pathway in neuropathic rats can reduce central sensitization [35].

Moreover, this study indicated a remarkable increase in spinal IL-6 level due to thymulin long-term treatment in CFA injected rats which it was aligned with hyperalgesia reduction due to thymulin administration. We measured lumbar spinal cord IL-6 during different stage of this study and our data revealed that spinal IL-6 level significantly decreased in parallel with microglia activity inhibition which indicated by Iba-1 expression variation by western blotting and Immunohistochemistry. Then, it seems there was relation between spinal microglia activity and IL-6 level during different phases of this study. According to our previous study, IL-6 is a multifunctional cytokine with pro and anti-inflammatory properties. As a matter of fact, IL-6 has a pro-inflammatory role in the acute phase and anti-inflammatory role during the chronic phase of CFA-induced inflammation. Some studies showed the anti-hyperalgesic effect of IL-6 during the chronic phase of study may be mediated by increased spinal mu opioid receptor expression [8,36]. Cytokines can be produced in the spinal cord. Microglia, astroglia and primary afferent fibers like DRG neurons are known sources of IL-6 [37]. Furthermore, Dardenne et al. showed that administration of low doses of thymulin (ng) can increase pro-inflammatory cytokines levels and resulted in hyperalgesia, however high effective doses (μg) of thymulin administration in accordance with our study have been shown to alleviate the inflammatory pain and reduce the up-regulated levels of cytokines [14]. Then, it seems that a part of the anti-inflammatory and anti-hyperalgesic effects of long-term thymulin administration may mediate via modulating the level of spinal pro/anti-inflammatory cytokines.

On the other hand, our results reveal that the administration of an effective dose of thymulin can inhibit the activation of lumbar spinal microglia which was clarified by immunohistochemistry staining. Then, reduced activity of spinal microglia not only can be responsible for hyperalgesia decrease due to thymulin treatment via decreased level of microglia related spinal cytokines but also can indicate anti-neuro inflammatory effects due to inhibition of some central signaling pathways. MAPKs intracellular signaling pathway is prominent intracellular mediators of signal transduction cascades in glia and causes to the genesis of chronic pain [12]. Some studies indicated that p38 MAPK signaling pathway is activated by pro-inflammatory cytokines in spinal microglia cells in the earliest phase of neuropathic pain [10]. Another study in our lab reported that administration of SB203580, by inhibiting of spinal p38 MAPK phosphorylation (Pp38) significantly alleviated hyperalgesia during CFA-induced inflammation [13]. In agreement with those reports, our study illustrated a significant increase in spinal levels of Pp38 MAPK during CFA-induced inflammation, which treatment by thymulin caused a meaningful reduction in the level of spinal Pp38 MAPK expression. It has been shown that thymulin treatment caused a prominent decrease in p38 phosphorylation in monocrotaline-induced pulmonary hypertension rats and it had an anti-inflammatory effect on lung diseases [38].

Moreover, by considering about the more potent anti-hyperalgesic and anti-inflammatory effects of thymulin than SB203580 treatment in CFA groups, it seems that these modulatory effects not only mediated by inhibition of p38 MAPK phosphorylation but also some other pathways which may related to inhibition of spinal microglia activity by thymulin can involve in those effects. Our data indicated that thymulin administration in CFA group caused more decrease in spinal Iba-1 level than CFA + SB203580 group. Nazemi et al. showed that microglia inhibition attenuated neuropathic pain by reducing Toll-like receptor 4 (TLR4) expressions in a chronic constriction injury (CCI) model of neuropathic pain in rats [39].

In summary, this study indicates that thymulin long-term treatment can be considered as an effective way to attenuate CFA-induced hyperalgesia and neuro-inflammation. These effects may be mediated by inhibition of spinal microglia activity and variation of production of some central inflammatory mediators such as cytokines by those cells. Also, it seems those anti-hyperalgesic and anti-inflammatory effects can be merged with the ability of thymulin in blocking of spinal p38 MAPK signaling pathway which needs more investigations. Of course studying the side effects of thymulin long term treatment will be useful for evaluation of its efficacy.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Phillips, K., Clauw, D.J., 2011. Central pain mechanisms in chronic pain states – maybe it is all in their head. Best Pract. Res. Clin. Rheumatol. 25, 141–154.
[2] Mika, J., Zychowska, M., Popiolek-Barczyk, K., Rojewska, E., Przewlocka, B., 2013. Importance of glial activation in neuropathic pain. Eur. J. Pharmacol. 716, 106–119.
[3] Hanisch, U.K., 2002a. Microglia as a source and target of cytokines. Glia 40, 140–155.
[4] Xu, B., Zhang, W.S., Yang, J.L., Lû, N., Deng, X.M., Xu, H., Zhang, Y.Q., 2010. Evidence for suppression of spinal glial activation by dexmedetomidine in a rat model of monoarthritis. Clin. Exp. Pharmacol. Physiol. 37.
[5] Gazerani, S., Zaringhalam, J., Manaheji, H., Golabi, S., 2016. The role of C fibers in spinal microglia induction and possible relation with TRPV3 expression during chronic inflammatory arthritis in rats. Basic Clin. Neurosci. 7, 231.
[6] B. Nasseri, V. Nazemian, H. Manaheji, J. Zaringhalam, Microglia are involved in pain related behaviors during the acute and chronic phases of arthritis inflammation, J. Cell. Mol. Anesth. 1 (2016) 137–145.
[7] Hanisch, U.K., 2002bb. Microglia as a source and target of cytokines. Glia 40, 140–155.
[8] E. Tekieh, J. Zaringhalam, H. Manaheji, N. Maghsoudi, B. Alani, H. Zardooz, Increased serum IL-6 level time-dependently regulates hyperalgesia and spinal mu opioid receptor expression during CFA-induced arthritis, EXCLI J. 10 (2011) 23–33.
[9] Hu, J.-H., Yang, J.-P., Liu, L., Li, C.-F., Wang, L.-N., Ji, F.-H., Cheng, H., 2012. Involvement of CX3CR1 in bone cancer pain through the activation of microglia p38 MAPK pathway in the spinal cord. Brain Res. 1465, 1–9.
[10] Jin, S.-X., Zhuang, Z.-Y., Woolf, C.J., Ji, R.-R., 2003. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. J. Neurosci. 23, 4017–4022.
[11] Svensson, C.I., Marsala, M., Westerlund, A., Calcutt, N.A., Campana, W.M., Freshwater, J.D., Catalano, R., Feng, Y., Protter, A.A., Scott, B., Yaksh, T.L., 2003. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. J. Neurochem. 86, 1534–1544.
[12] Ji, R.-R., Gereau, R.W., Malcangio, M., Strichartz, G.R., 2009. MAP kinase and pain. Brain Res. Rev. 60, 135–148.
[13] Zaringhalam, J., Tekieh, E., Manaheji, H., Akhtari, Z., 2013. Cellular events during

arthritis-induced hyperalgesia are mediated by Interleukin-6 and p38 MAPK and their effects on the expression of spinal mu-opioid receptors. Rheumatol. Int. 33, 2291–2299.
[14] Dardenne, M., Saade, N., Safieh-Garabedian, B., 2006. Role of thymulin or its analogue as a new analgesic molecule. Ann. N. Y. Acad. Sci. 1088, 153–163.
[15] Lunin, S.M., Novoselova, E.G., 2010. Thymus hormones as prospective anti-inflammatory agents. Expert Opin. Ther. Targets 14, 775–786.
[16] Saade, N.E., Atweh, S.F., Jabbur, S.J., Dardenne, M., Bach, J.F., Safieth-Garabedian, B., 2003. A thymulin analogue peptide with powerful inhibitory effects on pain of neurogenic origin. Neuroscience 119, 155–165.
[17] Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.
[18] Zaringhalam, J., Hormozi, A., Tekieh, E., Razavi, J., Khanmohammad, R., Golabi, S., 2014bb. Serum IL-10 involved in morphine tolerance development during adjuvant-induced arthritis. J. Physiol. Biochem. 70, 497–507.
[19] Zanjani, T.M., Sabetkasaei, M., Mosaffa, N., Manaheji, H., Labibi, F., Farokhi, B., 2006. Suppression of interleukin-6 by minocycline in a rat model of neuropathic pain. Eur. J. Pharmacol. 538, 66–72.
[20] Akhtari, Z., Zaringhalam, J., Eidi, A., Manaheji, H., Tekieh, E., 2012. Bidirectional effects of serum TNF alpha level and spinal P38MAPK phosphorylation on hyperalgesia variation during CFA-induced arthritis. EXCLI J. 11, 373.
[21] Zaringhalam, J., Akhtari, Z., Eidi, A., Ruhani, A.H., Tekieh, E., 2014aa. Relationship between serum IL10 level and p38MAPK enzyme activity on behavioral and cellular aspects of variation of hyperalgesia during different stages of arthritis in rats. Inflammopharmacology 22, 37–44.
[22] Zhao, X.H., Zhang, T., Li, Y.Q., 2015. The up-regulation of spinal toll-like receptor 4 in rats with inflammatory pain induced by complete Freund's adjuvant. Brain Res. Bull. 111, 97–103.
[23] Nagarkar, B., Jagtap, S., 2017. Effect of new polyherbal formulations DF1911, DF2112 and DF2813 on CFA induced inflammation in rat model. BMC Complement. Altern. Med. 17, 194.
[24] Singh, A.K., Vinayak, M., 2016. Anti-nociceptive effect of resveratrol during inflammatory hyperalgesia via differential regulation of pro-inflammatory mediators. Phytother. Res. 30, 1164–1171.
[25] Romero-Sandoval, E.A., Horvath, R.J., DeLeo, J.A., 2008. Neuroimmune interactions and pain: focus on glial-modulating targets. Current opinion in investigational drugs (London, England: 2000) 9, 726.
[26] M.R. Suter, Y.-R. Wen, I. Decosterd, R.-R. Ji, Do glial cells control pain? Neuron Glia Biol. 3 (2007) 255–268.
[27] Clark, A.K., Gentry, C., Bradbury, E.J., McMahon, S.B., Malcangio, M., 2007. Role of spinal microglia in rat models of peripheral nerve injury and inflammation. Eur. J. Pain 11, 223–230.
[28] Abbadie, C., Bhangoo, S., De Koninck, Y., Malcangio, M., Melik-Parsadaniantz, S., White, F.A., 2009. Chemokines and pain mechanisms. Brain Res. Rev. 60, 125–134.
[29] Ji, R.-R., Berta, T., Nedergaard, M., 2013. Glia and pain: is chronic pain a gliopathy? PAIN® 154, S10-S28.
[30] Nazemi, S., Manaheji, H., Zaringhalam, J., Sadeghi, M., Haghparast, A., 2012. Post-injury repeated administrations of minocycline improve the antinociceptive effect of morphine in chronic constriction injury model of neuropathic pain in rat. Pharmacol. Biochem. Behav. 102, 520–525.
[31] Shan, S., Hong, C., Mei, H., Ting-Ting, L., Hai-Li, P., Zhi-Qi, Z., Yu-Qiu, Z., 2007. New evidence for the involvement of spinal fractalkine receptor in pain facilitation and spinal glial activation in rat model of monoarthritis. Pain 129, 64–75.
[32] Yoon, S.-Y., Patel, D., Dougherty, P.M., 2012. Minocycline blocks lipopolysaccharide induced hyperalgesia by suppression of microglia but not astrocytes. Neuroscience 221, 214–224.
[33] Safieh-Garabedian, B., Mayasi, Y., Saadé, N.E., 2012. Targeting neuroinflammation for therapeutic intervention in neurodegenerative pathologies: a role for the peptide analogue of thymulin (PAT). Expert Opin. Ther. Targets 16, 1065–1073.
[34] Safieh-Garabedian, B., Dardenne, M., Pleau, J.M., Saade, N.E., 2002. Potent analgesic and anti-inflammatory actions of a novel thymulin-related peptide in the rat. Br. J. Pharmacol. 136, 947–955.
[35] Svensson, C.I., Schäfers, M., Jones, T.L., Powell, H., Sorkin, L.S., 2005. Spinal blockade of TNF blocks spinal nerve ligation-induced increases in spinal P-p38. Neurosci. Lett. 379, 209–213.
[36] P.E. Lipsky, Interleukin-6 and rheumatic diseases, Arthritis Res. Ther. 8 (Suppl. 2) (2006) S4.
[37] Vazquez, E., Kahlenbach, J., Segond von Banchet, G., König, C., Schaible, H.G., Ebersberger, A., 2012. Spinal interleukin-6 is an amplifier of arthritic pain in the rat. Arthritis Rheum. 64, 2233–2242.
[38] Henriques-Coelho, T., Oliveira, S.M., Moura, R.S., Roncon-Albuquerque Jr, R., Neves, A.L., Santos, M., Nogueira-Silva, C., La Fuente Carvalho, F., Brandao-Nogueira, A., Correia-Pinto, J., 2008. Thymulin inhibits monocrotaline-induced pulmonary hypertension modulating interleukin-6 expression and suppressing p38 pathway. Endocrinology 149, 4367–4373.
[39] Nazemi, S., Manaheji, H., Noorbakhsh, S.M., Zaringhalam, J., Sadeghi, M., Mohammad-Zadeh, M., Haghparast, A., 2015. Inhibition of microglial activity alters spinal wide dynamic range neuron discharge and reduces microglial toll-like receptor 4 expression in neuropathic rats. Clin. Exp. Pharmacol. Physiol. 42, 772–779.