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# Transforming growth factor-β-activated kinase 1 (TAK1) mediates chronic pain and cytokine production in mouse models of inflammatory, neuropathic, and primary pain

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## Abstract

The origin of chronic pain is linked to inflammation, characterized by increased levels of pro-inflammatory cytokines in local tissues and systemic circulation. Transforming growth factor beta-activated kinase 1 (TAK1) is a key regulator of pro-inflammatory cytokine signaling that has been well characterized in the context of cancer and autoimmune disorders, yet its role in chronic pain is less clear. Here, we evaluated the ability of our TAK1 small molecule inhibitor, takinib, to attenuate pain and inflammation in pre-clinical models of inflammatory, neuropathic, and primary pain. Inflammatory, neuropathic, and primary pain was modeled using intraplantar complete Freund's adjuvant (CFA), chronic constriction injury (CCI), and systemic delivery of the COMT inhibitor OR486, respectively. Behavioral responses evoked by mechanical and thermal stimuli were evaluated in separate groups of mice receiving takinib or vehicle prior to pain induction (baseline) and over 12 days following CFA injection, 4 weeks following CCI surgery, and 6 hours following OR486 delivery. Hindpaw edema was also measured prior to and 3 days following CFA injection. Upon termination of behavioral experiments, dorsal root ganglia (DRG) were collected to measure cytokines. We also evaluated the ability of takinib to modulate nociceptor activity *via* in vitro calcium imaging of neurons isolated from the dorsal root ganglia of Gcamp3 mice. In all three models, TAK1 inhibition significantly reduced hypersensitivity to mechanical and thermal stimuli and expression of pro-inflammatory cytokines in DRG. Furthermore, TAK1 inhibition significantly reduced the activity of tumor necrosis factor (TNF)-primed/capsaicin-evoked DRG

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nociceptive neurons. Overall, our results support the therapeutic potential of TAK1 as a novel drug target for the treatment of chronic pain syndromes with different etiologies.

**Keywords**

TAK1; Pain; inflammation; Neuropathic pain; primary pain

## Introduction

The complex etiology of chronic pain coupled with neuronal plasticity that underlies the development and maintenance of chronic pain syndromes has challenged our ability to identify effective treatments. Three distinct types of chronic pain including inflammatory, neuropathic, and primary pain, are characterized by diverse etiologic factors. Inflammatory pain results from activation and infiltration of immune cells in response to tissue damage or pathogen exposure, while neuropathic pain results from lesions to the peripheral or central nervous system.[53] Chronic primary pain (CPP) lacks a clear structural etiology, but is linked to heightened catecholaminergic tone characterized by systemic increases in epinephrine and norepinephrine alongside decreased activity of the catechol-O-methyltransferase (COMT) enzyme that metabolizes these catecholamines.[33]

Irrespective of the pain type or its initial cause, the chronification of pain is largely due to a heightened state of inflammation, characterized by increased levels of key pro-inflammatory mediators.[26,11] Acute pain following an injury or pathogen exposure is elicited by increases in pro-inflammatory cytokines that confer survival advantage by promoting immune responses that limit tissue damage and initiate tissue repair.[3, 16, 20] Sustained elevations in pro-inflammatory cytokines, however, elicit chronic pain which is maladaptive and has no biological significance. In particular, it has been shown that TNF is a key driver of chronic pain and inflammation.[37, 43, 44] TNF can bind to TNF receptor 1 (TNFR1) located on the terminals of pain-sensing primary afferent nociceptors to directly increase their activity.[14] In addition, TNF can stimulate its own production and that of other pro-inflammatory cytokines such as interleukin-17 (IL-17), interleukin-1β (IL-1β) and interferon gamma (IFNγ).[13] Patients with chronic pain exhibit increased levels of TNF, with higher levels seen in those with greater pain.[2, 4, 12, 18, 35]

Evaluation of the TNF-mediated pro-survival and inflammatory response pathway has revealed protein kinase TNFβ activated protein kinase 1 (TAK1) as a critical signaling node downstream of TNFR1 that plays a crucial role in mediating nuclear factor κ β (NF-kβ) as well as mitogen-activated protein kinases (MAPKs) important for inflammation and pain.[23, 24, 34, 40, 52] While numerous groups, including ours, have characterized the role of TAK1 in inflammation, very little is known about TAK1's role in pain.[41,51,54] In 2008, Katsura et al. demonstrated that TAK1 promotes astrocyte activation and mechanical pain, which is rescued by genetic knockdown of TAK1 in astrocytes.[25] Despite these promising findings, no pharmacological studies with selective TAK1 inhibitors have been performed.

Our group's recent advancements in drug discovery have identified a novel small molecule TAK1 inhibitor, takinib, which has high potency (~9 nM) and selectivity for TAK1.[51] In

previous studies, we demonstrated that takinib produced powerful anti-inflammatory effects in the absence of toxic side-effects.[51] Here, we assessed the analgesic and anti-inflammatory effects of TAK1 inhibition with takinib, in mouse models of inflammatory, neuropathic and primary pain.

## Methods

### Animal care

Male and female C57/bl6 mice 8-12 weeks old and approximately 16-21 grams were bred in-house or purchased from The Jackson laboratory (Bar Harbor, ME, USA). Mice were housed in a temperature and humidity-controlled facility under 12-hour light/dark cycle (lights on at 7 am) and access to food and water *ad libitum*. All experiments were approved and carried out in accordance of the Duke University Institution Animal Care and Use Committee (IACUC) and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

### General experimental design for in vivo studies

Here, we aimed to determine the role of TAK1 in mediating pain and cytokine production in three mouse models: the complete Freund's adjuvant (CFA) model of inflammatory pain, the chronic constriction injury (CCI) model of neuropathic pain, and the COMT inhibition model of primary pain. In the CFA model, male and female mice were randomly assigned to one of four experimental groups: 1) vehicle + CFA, 2) takinib + CFA, 3) vehicle + incomplete Freund's adjuvant (IFA), or 4) takinib + IFA. Takinib (50 mg/kg in 20mL volume) or vehicle was delivered daily subcutaneously (s.c.) over the course of 12 days beginning on day 1 immediately following CFA or IFA control. The dose of 50mg/kg takinib was chosen based on previous pharmacokinetic and efficacy studies.[39] Behavioral responses were determined at baseline and on days 1,2,3,5,7,10 and 12, 2 hours post takinib or vehicle delivery. A separate cohort of vehicle + CFA and takinib + CFA mice was used to measure DRG cytokines and hindpaw edema on day 3 following CFA.

In the CCI model, following male and female mice were randomly assigned to one of four experimental groups: 1) vehicle + CCI, 2) takinib + CCI, 3) vehicle + sham, or 4) takinib + sham. Takinib (50 mg/kg in 20 μL volume) or vehicle was delivered daily s.c. over the course of 2 weeks beginning on day 1 immediately following CCI or sham surgery. Behavioral responses were determined at baseline and at week 1, 2, 3, and 4, 2 hours post takinib or vehicle delivery. Upon termination of behavioral experiments, DRG were collected to measure cytokines at 4 weeks following CCI.

In the COMT inhibition model, male and female mice were randomly assigned to one of four experimental groups: 1) vehicle + OR486, 2) takinib + OR486, 3) vehicle + vehicle, or 4) takinib + vehicle. Takinib (50 mg/kg in 20 μL volume) or vehicle was delivered once s.c. immediately following OR486 or vehicle. Behavioral responses were determined at baseline and at 0.5, 1, 2, 3, 4, 5 and 6 hours post OR486. Upon termination of behavioral experiments, DRG were collected to measure cytokines at 6 hours following OR486. In

all three models, the experimenter was blinded to the treatment groups. Animals with heightened or abnormal baseline responses were removed from the study prior to initiation of disease induction and treatment.

### CFA model of inflammatory pain

The CFA model of chronic inflammatory pain is an experimental model that parallels human rheumatoid arthritis.[30] A volume of 25μL of CFA (Sigma Aldrich F5881) or Incomplete Freund's Adjuvant (IFA; as a control, Sigma Aldrich F5506) emulsified in a 1:1 0.9% sterile water was injected into the plantar surface of the gently restrained mouse's hindpaw which induces robust edema that peaks ~3 days post injection. Approximately 12.5 μg of heat killed Mycobacterium tuberculosis injected per paw.

### CCI model of neuropathic pain

The CCI model of chronic neuropathic pain is one of the most widely-used experimental models that parallels human conditions such as post-surgical pain, amputation pain, and chronic regional pain syndrome.[32] To induce CCI pain, a small incision was made along the mid-thigh of the mouse and muscle gently opened to expose the sciatic nerve. Four chromic gut sutures were loosely tied around the sciatic nerve just above the trifurcation. Then mice were sutured and monitored during recovery.

### COMT inhibition model of primary pain

The COMT inhibition model of primary pain developed in our lab employs the COMT inhibitor OR486 which leads to increased catecholamine levels, systemic inflammation, and multi-modal widespread pain similar to patients with these conditions.[5, 10, 19, 22, 29, 33, 36, 45, 46, 50, 55, 56] Mice were injected with the COMT inhibitor OR486 (30mg/kg) or vehicle i.p.

### Assessment of mechanical and thermal sensitivity

Prior to baseline recordings, all animals were handled and habituated to experimental apparatus for 5 days. Tactile sensory thresholds were determined by applying von Frey filaments (North Coast Medical, CA) to the plantar surface of the right hind paw and recording the withdrawal response using the up-down method.[17] Mice were placed in a clear Plexiglas chamber with a wire mesh floor and allowed to habituate for 30 minutes. Testing was conducted using filament sizes with respective bending g-forces of: 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 1.5 g (Stoelting, Wood Dale, IL, USA) starting at 0.16g force. Filaments were pressed upward into the soft spot of the left hind paw until the filament 'just bent' in half for a duration of 3 seconds. A response was recorded if the mouse expressed a lifting, shaking or licking behavior toward the hind paw during the application of the filament. Following initial response, a series of six total responses were recorded for each hind paw (ipsilateral and contralateral). Results were entered into a pre-programmed Excel sheet which uses a logarithmic algorithm to determine the gram-force value that would elicit paw withdrawal in 50% of trials ($10(Xf + k\delta)/10,000$, where Xf = value [in log units] of the final von Frey hair used; k = tabular value of positive and negative responses, and $\delta$ = mean difference [in log units] between stimuli). Mechanical allodynia was defined as a heightened

response to a normally innocuous stimulus, as determined by a decrease in paw withdrawal threshold.

Mechanical hyperalgesia was determined by measuring response frequency to a suprathreshold filament (1.5 g). The filament was applied to the hind paw 10 times for a duration of 1 s, with an interstimulus interval of 1s. The number of paw withdrawals (which could range from 0 to 10) was recorded for each hind paw at each time point. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals in response to a normally noxious mechanical stimulus.

Thermal heat and cold sensory thresholds were determined by assessing the latency of each mouse to respond to a noxious heat/cold source.[21] Animals were placed in individual Plexiglas chambers and habituated for approximately 10 minutes. After habituation, for thermal heat, a radiant beam of light was applied to the plantar surface of the rat hind paw through a glass floor heated to 30°C. For thermal cold latency, an approximate 1cm cube of dry ice was placed into an empty syringe and applied to the bottom of the Plexiglas below the mouse paw. Latencies of paw withdrawal from the heat stimulus were recorded in duplicate. If the second paw withdrawal latency was not within ±4seconds of the first withdrawal latency, then a third measure was recorded. The 2 latencies closest in value were averaged and included in the analysis. Thermal hyperalgesia was defined as a decrease in paw withdrawal latency to a noxious thermal stimulus compared to baseline.

### Edema measurements

To determine hindpaw edema, paw diameter (mm) was measured using a caliper (Mitutoyo 530-101 Vernier Calipers). Measurements were taken at baseline and 3 days post CFA approximately 3-4 hours post treatment with takinib or vehicle.

### Cytokine analysis

Lumbar L2-L5 DRG were isolated from individual animals and lysed in lysis buffer containing 10% Triton X-100, 0.5M Tris HCL pH 7.4, 0.2M NaCl, 0.1M EDTA, protease and phosphorylase inhibitors and 10μL of 1M DTT for 20 minutes on ice. Protein concentration for all samples was measured using the Bradford assay, and then 300 μg of protein loaded onto an R&D mouse cytokine proteome array (ARY006) as per manufacture protocol. Data were captured by chemiluminescence on x-ray film, images inverted and analyzed by ImageJ software.

### Calcium imaging

Ca2+ imaging was performed in mouse DRG neurons isolated from Pirt-GCaMP3 mice.[27] DRG were removed aseptically from euthanized mice and incubated with collagenase (1.25 mg/mL)/dispase-II (2.4 units/mL) at 37°C for 120 min, then digested with 0.25% trypsin for 8 min at 37°C, followed by 0.25% trypsin inhibitor. Cells were mechanically dissociated with a flame polished Pasteur pipette in the presence of 0.05% DNase I. DRG cells were plated on glass coverslips and grown in a neurobasal defined medium with 2% B27 supplement and 5 mM AraC maintained in a 5% CO2 incubator at 37°C. DRG neurons were grown for 24 hr before use. The imaging buffer included 140 mM NaCl, 10

mM D-(+)-Glucose, 1 mM MgCl2, 2 mM CaCl2, 5 mM KCl, 10 mM HEPES, pH = 7.4, osmolarity = 320 mOsm/L. Prior to recording, cells were treated with vehicle or takinib (10μM) overnight. Cells were treated with TNF at 10ng/mL concentration or vehicle from 60-180 seconds. Calcium signals were measured under 20X lens using green emitted light in a 3 s interval. For each cell, the pixel intensity (Ft) was assessed for each frame and the pixel intensity recorded from the first 10 frames was taken to determine the average baseline value (F0). Ca2+ signal amplitudes are presented as ΔF/F0, which is the ratio of fluorescence difference (Ft-F0) to baseline value (F0). Positive responding cells were defined as those showing a >20% in fluorescence intensity from baseline values.

### Statistical Analysis

Group differences in behavioral responses, cytokines and peak Ca2+ intensity were analyzed by 1- or 2-way ANOVA. *Post hoc* comparisons were performed using the Bonferroni test, which corrected for multiple comparisons. Group differences in paw edema were analyzed by t-test. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

## Results

### Effects of TAK1 inhibition on inflammatory, neuropathic and primary pain

We evaluated the analgesic efficacy of the selective TAK1 inhibitor takinib in the CFA model of inflammatory pain, the CCI model of neuropathic pain, and the COMT inhibition model of primary pain. In the CFA model, male and female mice developed pronounced and persistent hypersensitivity to mechanical and thermal heat stimuli lasting 7-12 days (Figure 1A–C), while those in the IFA control group did not (Figure S1A–C). Compared to mice receiving vehicle, those receiving takinib immediately following CFA, exhibited reduced mechanical allodynia ($F_{1,196}$=82.65, P<0.0001) and mechanical hyperalgesia ($F_{1,196}$=26.85, P<0.0001). Interestingly, those animals receiving takinib showed hyposensitivity to thermal heat ($F_{1,205}$=84.65 P<0.0001). Of note, mice in the IFA control group receiving takinib also exhibited a modest reduction in thermal heat hyperalgesia compared to those receiving vehicle ($F_{1,175}$=10.94, P<0.0011) (Figure S1C).

In the CCI model, male and female mice developed pronounced hypersensitivity to mechanical and cold stimuli lasting up to 28 days (Figure 1D–F), while those in the sham control group did not (Figure S1D–F). Administration of takinib daily for the first 14 days attenuated mechanical allodynia ($F_{1,150}$=45.34, P< 0.0001) and mechanical hyperalgesia ($F_{1,90}$=15.32, P<0.001) with no significant effect observed in thermal cold hyperalgesia ($F_{1,90}$=2.43, P=0.123) (Figure 1 D–F). No differences in mechanical or thermal sensitivity were observed sham control group mice receiving takinib or vehicle (Figure S1 D–F).

In the COMT inhibition model, male and female mice developed hypersensitivity to mechanical and thermal heat stimuli within 30 minutes that persisted for 6 hours (Figure 1G–I), while those receiving vehicle did not (Figure S1G–I). Administration of takinib attenuated mechanical allodynia ($F_{1,165}$=9.33, P<0.002) and thermal heat hyperalgesia ( $F_{1,65}$=21.93, P<0.001). Notably, mice in the vehicle control group receiving takinib also

exhibited a modest reduction in mechanical allodynia compared to those receiving vehicle ($F_{1,176}$=8.55, P<0.003) (Supplemental Figure 1G). Finally, we tested the effects of takinib (50mg/kg, QD, s.c.) on locomotor activity using the rotorod test. No differences in time spent on the rotorod were found between mice treated with takinib compared to vehicle (Supplemental Fig. 2).

## Effect of TAK1 inhibition on DRG cytokine levels in inflammatory, neuropathic and primary pain models

Next, we evaluated the anti-inflammatory effects of takinib in L2-L5 DRG collected from mice in the three pain models. DRG were collected upon termination of behavioral experiments and levels of 40 cytokines measured by proteome array. In all three models, TAK1 inhibition with takinib reduced the pro-inflammatory milieu consistent with previous in vitro and in vivo work (Figure 2A, Figure 3S). In the CFA model, takinib significantly reduced expression levels of the following 16 cytokines: CCL1 (2.05-fold, P<0.001), IFNγ (1.5-fold, P<0.005), IL-1β by (1.95-fold, P<0.04), IL-7 (1.6-fold ,P<0.03), IL-13 (2.07-fold, P<0.02), IL-16 (1.88-fold, P<0.003), CXCL10 (1.7-fold, P<0.005), CXCL11 (1.32-fold , P=0.03), CCL12 (2.5-fold, P<0.001), CXCL9 (2.58, P<0.02), CCL3 (3.05-fold, P<0.004), CXCL2 (2.3-fold, P<0.04), CXCL12 (1.03-fold, P<0.04), CCL17 (1.22-fold , P<0.04), TIMP-1 (1.3-fold, P<0.006), and TREM-1 (2.05-fold, P<0.05). Furthermore, analysis of TNF expression on day 2 in the CFA model demonstrated that takinib treatment significantly reduced CFA-induced increases in TNF serum levels (P<0.0001) (Figure 2B).

In the CCI model, takinib significantly reduced expression levels of the following 4 cytokines: CCL11 (2.4-fold, P<0.01), IL-1r (1.69-fold, P<0.05), IL-2 (2.16-fold, P<0.002), and CCL17 (1.7- fold, P<0.002).

In the COMT inhibition model, takinib reduced expression levels of the following 13 cytokines: G-CSF (1.51-fold, P< 0.02), CCL1 (2.56-fold, P<0.001), IL-4 (1.45-fold, P<0.02), IL-7 (1.42-fold, P=0.05), IL-13 (1.58-fold, P<0.05), CXCL10 (1.44-fold, P<0.05), CXCL11 (1.36-fold, P<0.02), M-CSF (1.35-fold, P<0.02), CCL2 (1.56-fol, P<0.01), CCL12 (2.27-fold, P<0.01), CXCL9 (1.58-fold, P<0.03), CCL3 (1.72-fold, P<0.04), and TIMP-1 (1.29-fold , P<0.03).

Notably, takinib targeted 9 of the same cytokines in the CFA and COMT models: CCL1, IL-7, IL-13, CXCL10, CXCL11, CCL12, CXCL9, CCL3, and TIMP-1. In both the CFA and CCI models, takinib targeted CCL17.

## Effect of TAK1 inhibition on CFA-induced paw edema

CFA induces robust edema in the inflicted paw, which peaks 3 days post injection and can persist for weeks. Paw edema was evaluated by paw caliper measurements at baseline and on day 3 following administration of CFA with vehicle or takinib treatment. Compared to mice receiving vehicle, those receiving takinib exhibited significant reductions in CFA-induced edema ($T_{ratio}$=3.224, P<0.003) (Figure 3).

### TAK1 inhibition blocks TNF-induced increases in neuronal activity

As TAK1 is expressed in nociceptors, we next sought to determine the effects of takinib on the activity of DRG nociceptive neurons. Nociceptors isolated from Gcamp3 mice were acutely cultured and treated with either takinib or vehicle prior to TNF priming and nociceptor activation with capsiacin. Vehicle+Vehicle treated neurons showed a robust calcium influx in response to the TRPV1 agonist capsaicin, whereas Vehicle+TNF primed nociceptors showed a heightened ($p=0.047$) response to capsaicin (Figure 4A, B). Pre-incubation of nociceptors with takinib blocked the TNF-induced increases in calcium response ($P<0.004$). Not only did takinib block the average calcium influx in response to TNF and capsaicin, but it also reduced the number of nociceptors responding to the capsaicin challenge from ~42% to 34% (Figure 4C). Based on our findings we posit that TAK1 inhibition blocks TNF and other inflammatory mediators from upregulating NF-κβ signaling pathways in nociceptors leading to a reduced pain state (Figure 5).

## Discussion

Conventional therapies, such as non-steroidal anti-inflammatory drugs (NSIADs), opioids and antidepressants, have poor efficacy for managing chronic pain as well as adverse side-effects, including heart attack, stroke, altered mental state, addiction, and life-threatening respiratory depression.[7] Thus, it is urgent that we investigate previously unexploited targets for the development of safer, more effective treatments. As a key mediator of cytokine and MAPK signaling events underlying the onset and maintenance of a variety of pain conditions, TAK1 may represent such a target. Yet, the role of TAK1 in pain remains unclear. Thus, we conducted a set of pharmacologic studies to evaluate the therapeutic potential of TAK1 inhibitors for distinct types of pain. Here, we provide the first demonstration that the selective TAK1 inhibitor takinib attenuates mechanical and thermal pain and reduces DRG cytokine levels in models of inflammatory, neuropathic and primary pain. Furthermore, we show that takinib reduced capsaicin-induced activity of nociceptive neurons primed with the pro-inflammatory cytokine TNF.

First, we examined the effects of targeted TAK1 inhibition on pain in three distinct pre-clinical mouse models. The CFA, CCI and COMT models have all been shown to have varying levels of proinflammatory cytokine expression following disease onset, and NF-κβ and downstream p38/JNK pathways have also been shown to be active and essential to the development of pain in these models.[9,22]Takinib alleviated mechanical hypersensitivity in all three models. Takinib also alleviated thermal heat hypersensitivity in inflammatory and primary pain models, with a reduction of thermal cold hypersensitivity during takinib treatment (first 14 days) in the neuropathic pain model. Further, takinib had analgesic effects in control mice, such that it reduced baseline paw withdrawal latencies to mechanical stimuli or thermal heat in mice receiving OR486 or IFA, respectively. Although studies on TAK1 and chronic pain are limited, one group found that reduced phosphorylated TAK1 in the DRG and sciatic nerve corresponded to reduced CCI-induced neuropathic pain.[15] Additionally, expression of TAK1 in astrocytes in the CNS has been shown to regulate nerve-injury induced mechanical hypersensitivity.[25] Together, these data suggest that TAK1 contributes to normal nociceptive as well as maladaptive persistent pain.

As TAK1 is a key regulator of pro-inflammatory signaling pathways that underlie pain, we next sought to characterize the effects of TAK1 inhibition on cytokine profiles in DRG isolated from mice in the three pain models. Of the 40 cytokines measured, takinib reduced levels of 16 in the inflammatory pain model and 13 in the primary pain model, with 9 common to both models. The upregulation of key pro-inflammatory signaling cytokines and chemokines such as IL-6, TNF and CCL's and CXCL's have been observed in both the CFA inflammatory and COMT primary pain models. In the CFA model, the upregulation of inflammatory cytokines and chemokines occurs shortly after disease onset (1-3 days) and corresponds with increased pain behaviors.[8] In the primary pain model, the COMT inhibitor OR486 has been shown to upregulate the expression if IL-6, IL-1β and TNF signaling within 6 hours of inhibition. The rapid and acute upregulation of inflammatory cytokine signaling in the COMT model has been shown to be regulated by MAPK and NFκβ activation. In the case of chemokine expression in the DRG, upregulation has been shown to increase the migration of immune cells (i.e macrophages and T cells) into the DRG leading to increased expression of inflammatory cytokines and subsequent hyperactivity of nociceptors. [1, 31, 47, 49] To this end, we have previously shown that TAK1 inhibition can reduce the expression of pro inflammatory mediators such as TNF, IL-6, IL-1β, IL-2 and chemokines such as CCL1, CCL2, CCL3 and CXCL's in immune cells stimulated with TNF or LPS.[38–42] Based on these results, we believe that takinib is able to block the upregulation of key chemokines in the DRG in the CFA and COMT models and prevent the development of immune mediated mechanical and thermal hyperalgesia.

Meanwhile, in the CCI neuropathic pain model we observed that takinib reduced levels of 4 pro-inflammatory cytokines 4 weeks post CCI surgery. Similar to the inflammatory and primary pain models, previous research has characterized the robust expression of inflammatory cytokines and chemokines in the DRG following CCI disease onset. For example, the expression of inflammatory mediators such as TNF and IL-1β, have been observed up to 4 weeks post CCI surgery in the DRG.[6, 28, 43] The persistent expression of these inflammatory mediators has been shown to regulate the length and duration of neuropathic pain and in human patients, correlate to pain intensity.[18] Similar to the inflammatory and primary pain models, we observed that takinib treatment downregulated CCL11, CCL17, IL-1r, and IL-2 in the CCI model. While several differences in cytokine expression were observed 4 weeks post CCI surgery, these findings might be limited due to the timing of cytokine collection in the DRG. Takinib produced significant analgesia over the course of the 2 week delivery period, after which pain levels increased and were comparable to those observed in vehicle-treated mice. Thus, measurement of inflammatory cytokine levels within 2 weeks post CCI during takinib delivery may have revealed greater group differences.

Based on our cytokine analyses in all three models, we believe that inhibition of TAK1 interrupts the expression and migration of immune cells into the DRG via inflammatory cytokines and chemokines. Furthermore, previous data collected by our lab and others have shown that TNF expression is affected by TAK1 inhibition. To better study the effects of takinib on TNF expression in the CFA model we measured serum TNF levels on day 2 of the CFA model. TNF expression has been shown to be greater in the initial days post CFA where is plays a central role in regulating proinflammatory responses. [38, 52] Our results

support that TAK1 inhibition may block the early expression of TNF in the CFA model preventing downstream immune activation and neuroinflammation. Together, these results demonstrate that TAK1 plays a central role in regulating inflammatory signaling pathways common across distinct types of pain.

As inflammation has been shown to augment NF-kβ and MAPK signaling in DRG nociceptive neurons, we conducted a final set of in vitro Ca2+ imaging studies to elucidate the effects of TAK1 inhibition on nociceptor activity. We found that TNF enhanced capsaicin-induced nociceptor responses, and that the effect was completely blocked by takinib. We hypothesize that takinib reduces DRG nociceptor activity by decreasing inflammatory cytokine and chemokine signaling. Previous groups have defined the role of TNF in priming nociceptors and enhancing their activity in response to stimuli.[48] Furthermore, our group has shown that TNF stimulation leads to downstream TAK1 phosphorylation which can be blocked with takinib.[51] Thus, we believe that takinib treatment attenuates TNF mediated nociceptor hyperactivity leading to a reduction in behavioral pain. These results are concurrent with our behavioral assays and cytokine data showing reductions in inflammatory cytokines and pain behaviors.

Based on our data in three pre-clinical models of pain, we posit that small molecule drugs that selectively target TAK1 may represent a safer and more effective option for long-term management of chronic pain. To date, no TAK1 inhibitors have advanced to phase I clinical studies. Other TAK1 inhibitors have been described in the literature (i.e. 5Z)-7-oxozeaenol (5ZO) and NG-25), however these have shown off target liabilities that confound interpretation of results.[52] To this end, our group discovered the takinib chemical scaffold and have characterized its potent and selective nature within the human purinome/kinome.[40] Although potent and selective, a current limitation to takinib is its lack of oral bioavailability which would be clinically relevant in the context of chronic pain patients. Future structure activity relationship (SAR) analog development is currently ongoing to discover an orally bioavailable takinib analog that represents a stronger clinical candidate. Despite this limitation, our data with takinib support that TAK1 is a novel pharmacological target for the treatment of chronic pain.

Our results support that TAK1 serves as a critical signaling node in this priming pathway and that inhibition can prevent TNF-nociceptor priming. Both pre-clinical and clinical data support that inflammatory priming events that underlie the development of chronic pain are critical to the development and maintenance of chronic pain conditions. Thus therapies, such as takinib, may prevent these priming events leading to reduced pain in patients. Future studies will further explore the ability of TAK1 targeted therapies to reverse chronic pain in established models.

## Conclusion

Our findings suggest that TAK1 plays an integral role in coordinating pro-inflammatory signaling cascades that promote nociception and pain, irrespective of the precipitating cause. Thus, TAK1 targeted therapies may provide broad acting relief for different types of acute and well as chronic pain conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Perspective:**

This article reports the therapeutic potential of TAK1 inhibitors for the treatment of chronic pain. This new treatment has the potential to provide a greater therapeutic offering to physicians and patients suffering from chronic pain as well as reduce the dependency on opioid based pain treatments.

## Highlights

- Chronic pain conditions lack effective long term therapeutics
- TAK1 is a novel drug target that attenuates pain in 3 preclinical pain models
- Takinib is a selective small molecule inhibitor of TAK1
- TAK1 inhibition prevents the expression of several key inflammatory cytokines

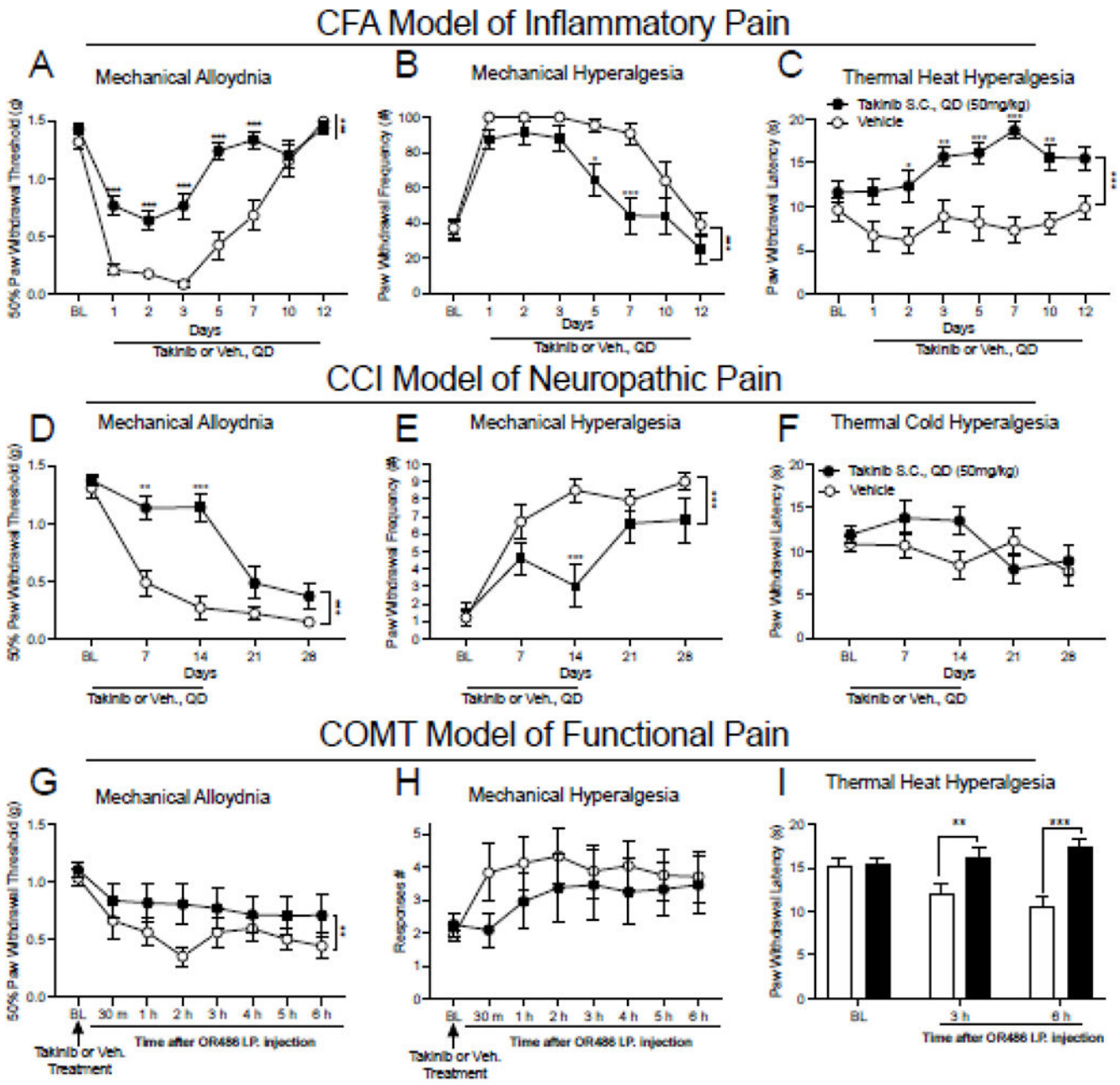


**Figure 1. Takinib reduces hypersensitivity to mechanical and thermal stimuli in three distinct models of pain.**

Compared to mice receiving vehicle, those receiving takinib exhibited reduced mechanical allodynia, mechanical hyperalgesia, and thermal heat hyperalgesia induced by CFA (A-C), sciatic nerve ligation (D-F), or systemic COMT inhibition with OR486 (G-I). N=12 mice (6M + 6F) per group. Data are shown as Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 different from vehicle.

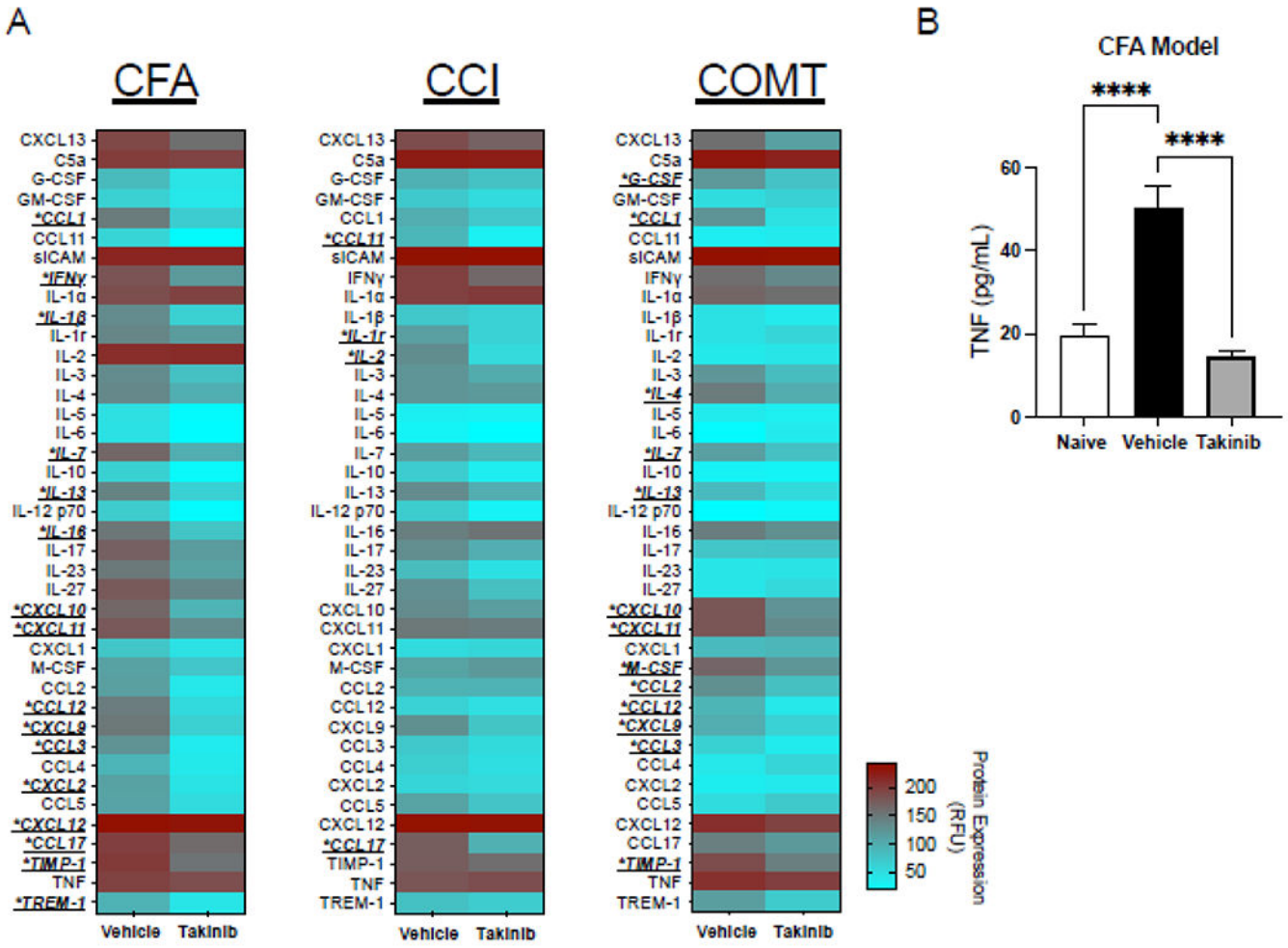


**Figure 2. Takinib reduces cytokine levels induced by CFA, CCI, or COMT inhibition.**

Data presented at relative fluorescent units (RFU) of protein expression, red coloring represents higher expression compared to light blue (lower expression). N=8 (4M + 4F) mice per group. *P<0.05, different from vehicle treatment (A). Serum TNF expression on day 2 of CFA model. N=8 (4M+4F) mice per group. Data shown as Mean±SEM. ****P<0.0001 different from vehicle control (B).

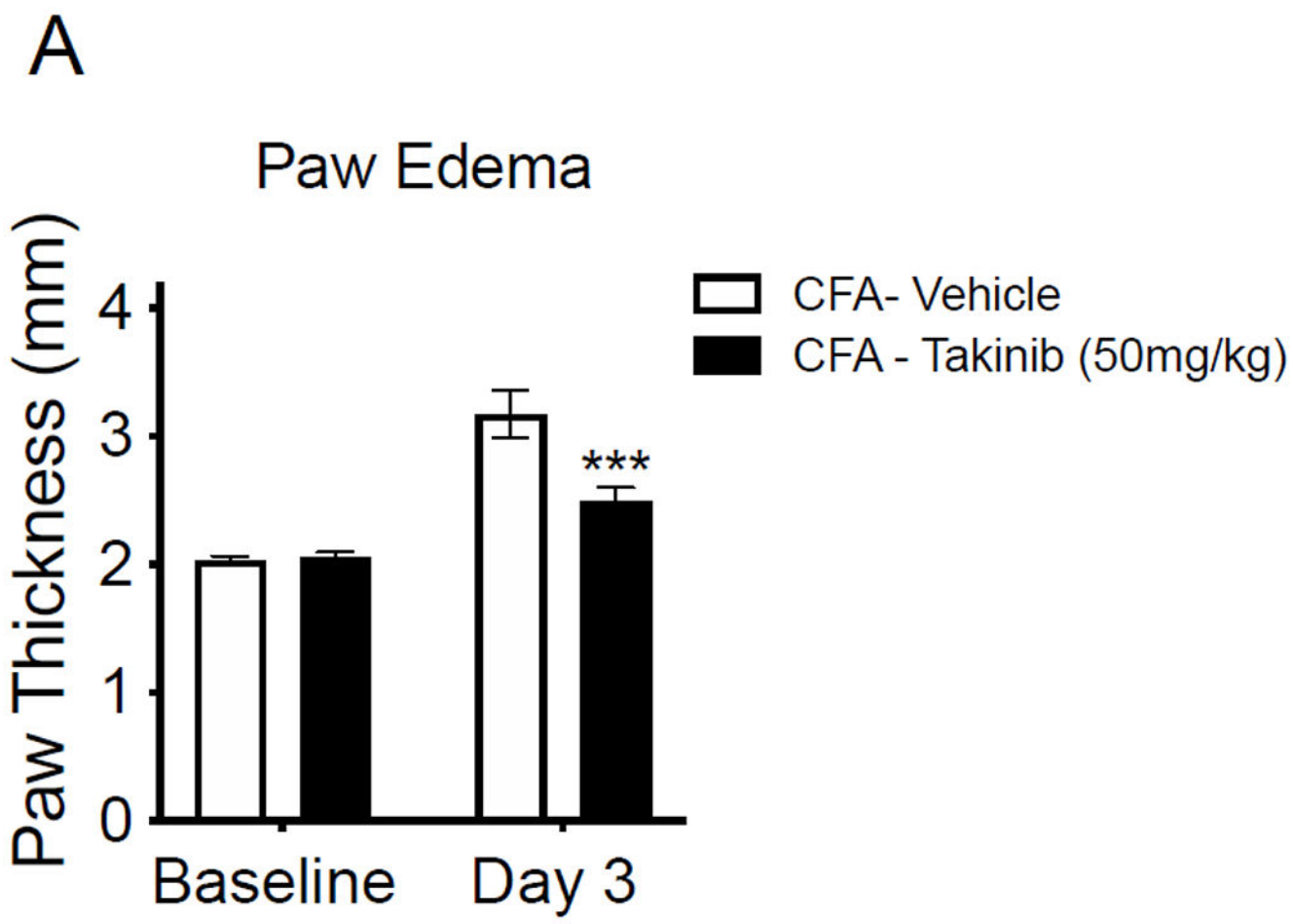


**Figure 3. Takinib reduces CFA-induced increases in paw diameter.**

N=13-15 mice (8-7M and 6-7F) per group. Data are shown as Mean ± SEM. ***P<0.001 different from vehicle.

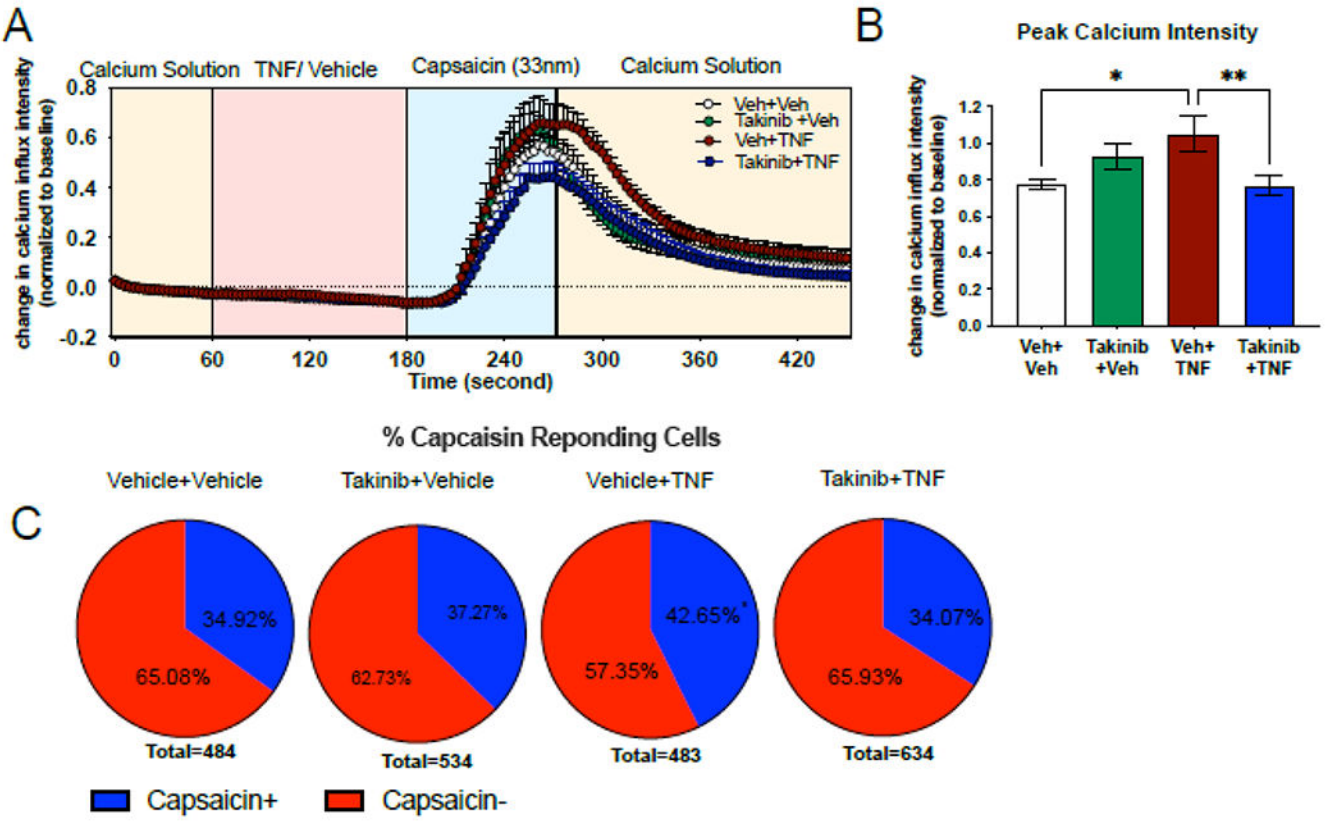


**Figure 4. TAK1 inhibition reduces TNF-induced increases in nociceptor activity.**

Pretreatment of nociceptive neurons with TNF induced increased responses to capsaicin applied at 180 s, as shown in the trace timeline (A) and the time corresponding to peak Ca2+ intensity (B). The corresponding percentages of capsaicin positive responding nociceptors is also shown (C). N=6-8/cells per treatment group.

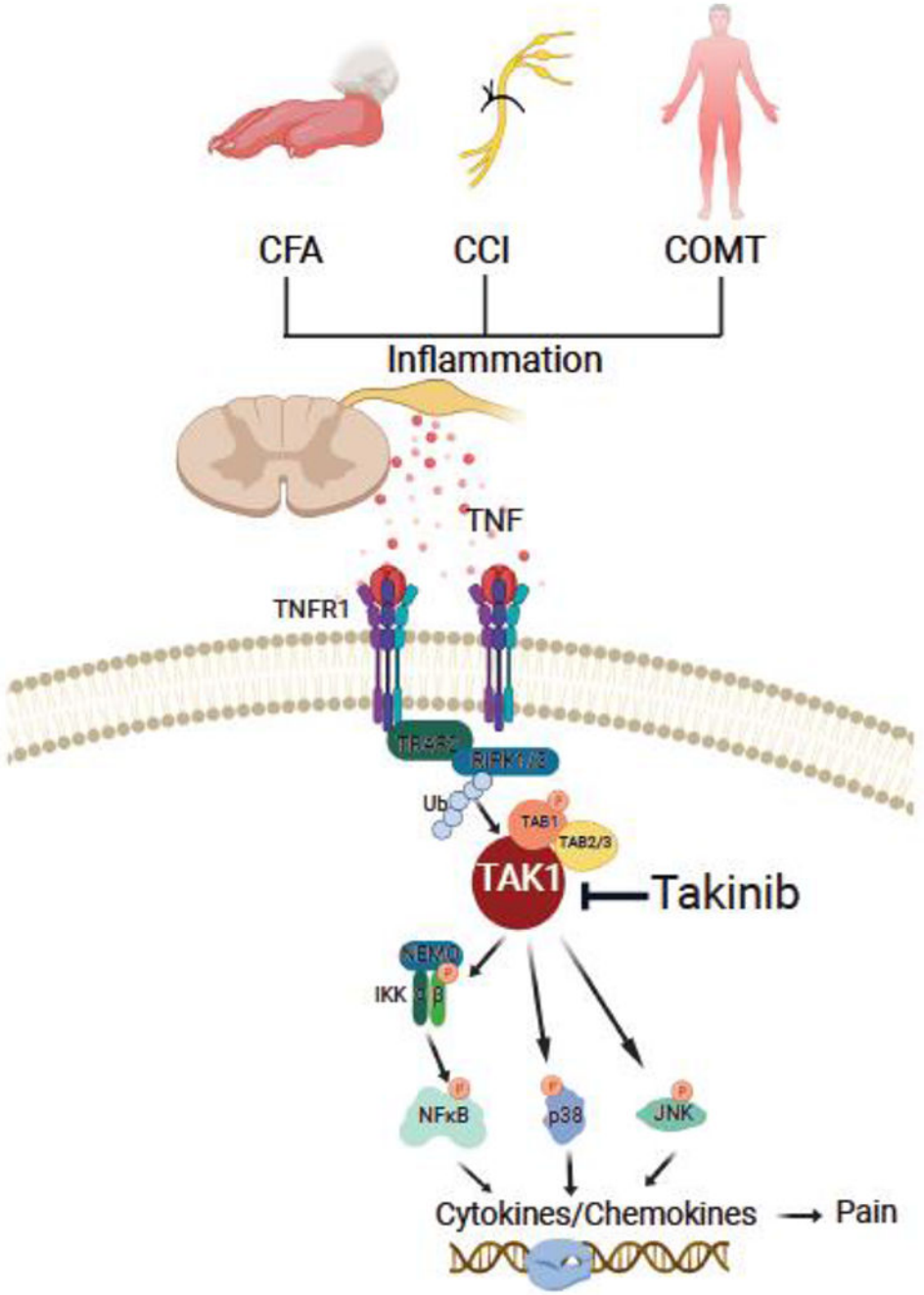


**Figure 5. Schematic of TAK1 signaling in nociceptors.**

Following binding of TNF to TNFR1 on nociceptors and immune cells, TAK1 phosphorylation by TRAF2/RIPK1/3 activates TAK1 leading to downstream activation of NF-κβ and transcription of pro inflammatory cytokines. Inhibition of TAK1 by takinib, blocks downstream inflammatory cytokines/chemokine expression and prevents the development of pain.