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RESEARCH PAPER

A store-operated calcium channel inhibitor attenuates collagen-induced arthritis

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BACKGROUND AND PURPOSE

Store-operated calcium (SOC) channels are thought to play a critical role in immune responses, inflammatory diseases and chronic pain. The aim of this study was to explore the potential role and mechanisms of SOC channels in collagen-induced arthritis (CIA).

EXPERIMENTAL APPROACH

The CIA mouse model was used to examine the effects of the SOC channel inhibitor YM-58483 on CIA and arthritic pain. Hargreaves' and von Frey hair tests were conducted to measure thermal and mechanical sensitivities of hind paws. ELISA was performed to measure cytokine production, and haematoxylin and eosin staining was used to assess knee histological changes. Western blot analysis was performed to examine protein levels.

KEY RESULTS

Pretreatment with 5 or 10 mg·kg^{-1} of YM-58483 reduced the incidence of CIA, prevented the development of inflammation and pain hypersensitivity and other signs and features of arthritis disease. Similarly, treatment with YM-58483 after the onset of CIA: (i) reversed the clinical scores; (ii) reduced paw oedema; (iii) attenuated mechanical and thermal hypersensitivity; (iv) improved spontaneous motor activity; (v) decreased periphery production of IL-1β, IL-6 and TNF-α; and (vi) reduced spinal activation of ERK and calmodulin-dependent PKII (CaMKIIα).

CONCLUSIONS AND IMPLICATIONS

This study provides the first evidence that inhibition of SOC entry prevents and relieves rheumatoid arthritis (RA) and arthritic pain. These effects are probably mediated by a reduction in cytokine levels in the periphery and activation of ERK and CaMKIIα in the spinal cord. These results suggest that SOC channels are potential drug targets for the treatment of RA.

Abbreviations

CaMKIIα, calmodulin-dependent protein kinase IIα; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; SOC channels, store-operated calcium channels; SOCE, store-operated calcium entry; YM-58483, 4-methy-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide

Tables of Links

TARGETS	
Ion channels[a]	**Enzymes**[b]
TRPC	ERK1
TRPM4	ERK2
	JNK
	p38

LIGANDS	
IL-1β	TNF-α
IL-6	YM-58483
LPS	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease that causes joint destruction and pain. It is believed that the primary cause is associated with activation of immune cells. Inflammation is driven either by B-cell or T-cell products stimulating the release of TNF-α and other cytokines, which contribute to the development and progression of RA. Although peripheral inflammation is the basis of RA, pain is the most common symptom of RA at all stages and adversely affects the patient's quality of life. Pain hypersensitivity is not always correlated with inflammation intensity and for many patients, pain is not relieved upon the reduction of inflammation (Lee, 2013). It has been shown that mechanical pain thresholds at both primary (joint) and secondary (non-joint) sites are significantly lower in RA patients than in healthy controls (Gerecz-Simon *et al.*, 1989), suggesting that not only peripheral sensitization, but also central sensitization contributes to arthritic pain. Similarly, in the collagen-induced arthritis (CIA) mouse model of RA, pain hypersensitivity occurs before inflammation develops (Inglis *et al.*, 2007; Bas *et al.*, 2012), further suggesting alterations in central pain sensitization. Taken together, these clinical and experimental observations strongly support the view that arthritic pain is the result of a complex physiological and pathological interaction between central sensitization and peripheral inflammation (Phillips and Clauw, 2013). The primary treatments for arthritic pain, such as anti-inflammatory drugs and biological agents, have historically focused on reducing peripheral inflammation (Scott *et al.*, 2010). More recently, attention has been directed towards treating the pain associated with RA (Lee, 2013), but there are very few treatments specifically designed to relieve arthritic pain. Therefore ongoing research is dedicated to identifying novel therapeutic targets that, ideally, alleviate both the peripheral inflammation and pain associated with RA.

It is well known that sustained Ca^{2+} entry is critical for responses initiated by activation of T-cells and B-cells, including proliferation and cytokine production (Di Sabatino *et al.*, 2009; Parekh, 2010). Store-operated calcium (SOC) channels are highly Ca^{2+}-selective cation channels that can be activated by depletion of endoplasmic reticulum (ER) calcium stores. SOC channel calcium entry (SOCE) is a major mechanism for triggering Ca^{2+} influx in immune cells (Vig and Kinet, 2009). There has been an explosive growth in establishing the molecular mechanisms that mediate SOCE and the role of this process in normal cellular function and disease states. The first molecular component of the system, Orai1, was identified and cloned in 2006 and it is now clear that the system is comprised of three structurally related pore-forming subunits (Orai1,2,3) located in the plasma membrane and two calcium sensors (stromal interaction molecules STIM1,2) located on the surface of the ER (Lewis, 2007). SOCE is implicated in several disorders including allergies, multiple sclerosis, cancer and inflammatory bowel disease (Ma *et al.*, 2010; McCarl *et al.*, 2010). One of the more important functions mediated by SOC channels is the control of cytokine production in immune cells. It has been shown that YM-58483, a potent inhibitor of SOC channels, blocks SOCE in immune cells and reduces cytokine production from these cells (Zitt *et al.*, 2004). Previous studies have also shown that YM-58483 inhibits red blood cell-induced delayed-type hypersensitivity responses and reduces antigen-induced bronchial asthma (Yoshino *et al.*, 2007; Ohga *et al.*, 2008b). Consistent with these peripheral actions, we have recently demonstrated that YM-58483 reduces oedema and local levels of TNF-α and IL-1β in an inflammatory pain model (Gao *et al.*, 2013).

Persistent pain or inflammation, such as that associated with RA, has been shown to lead to a phenomenon called central sensitization. This is a well-established form of synaptic plasticity that involves an enhancement of the functional state of neurons and circuits in nociceptive pathways in the CNS. It results from increased membrane excitability and synaptic efficacy as well as reduced inhibition and produces a state of heightened sensitivity in which noxious stimuli are amplified and previously subthreshold stimuli become noxious (Latremoliere and Woolf, 2009). There is growing evidence that central sensitization contributes to the pain experienced by patients with RA (Meeus *et al.*, 2012; Walsh and McWilliams, 2012), suggesting that an effective therapeutic should target this mechanism as well as peripheral inflammation. We have previously shown that YM-58483 has the properties of a broad spectrum analgesic and inhibits the hypersensitivity associated with neuropathic pain (Gao *et al.*, 2013).

Given the important roles of SOC channels in inflammation and pain, we hypothesized that inhibition of SOC channels would attenuate both the pathological and behavioural manifestations of CIA and arthritic pain. Here, we demonstrated that YM-58483 significantly decreases the clinical

score and the histological joint inflammation, reduces mechanical and thermal pain hypersensitivity, and improves deficits in spontaneous movement in CIA mice. These findings indicate that inhibition of SOC channels has anti-arthritis and analgesic actions and these channels may represent viable therapeutic targets for the treatment of RA.

Methods

Animals

Adult male DBA/1 mice, age 7–8 weeks, were used for all studies. Experiments were done in accordance with the guidelines of the National Institutes of Health and the Committee for Research and Ethical Issues of IASP and were approved by the Animal Care and Use Committee of Drexel University College of Medicine. Mice were housed in a room with a 12 h/12 h light/dark cycle, and habituated in the testing room 2–3 days before experiments. All experiments were performed with the experimenter blind to the drug treatments. A total of 70 animals was used in the experiments described here. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Induction of arthritis

The CIA model is induced by immunization as described previously (Luo *et al.*, 2011). Briefly, 100 μg chicken type II collagen (CII) was mixed with 100 μL complete Freund's adjuvant and injected i.d. on day 0 at the base of the tail. On day 21, a booster injection of 100 μg chicken CII in 100 μL of PBS was administered i.p. On day 28, 5 μg of lipopolysaccharide (LPS) was injected i.p. to synchronize disease onset. Normal mice were injected with PBS on days 0, 21 and 28.

Assessment of arthritis

The severity of arthritis was determined by a visual score of 0–4 per paw and summed for all four paws (i.e. maximum score of 16 per animal) according to the following criteria: 0 = normal, 1 = erythema and swelling in the digits, 2 = erythema and swelling in the mid-hind paw/mid-fore paw, 3 = erythema and swelling in the ankle/wrist, and 4 = erythema and severe swelling of the entire paw, including joint rigidity. Scores were determined twice a week for the entire duration of the experiment. Paw volume was measured before CII injection to establish the baseline, and twice a week after CIA developed using a modified plethysmometer as described previously (Gao *et al.*, 2013). Volume change was determined by subtraction of baseline from post-CIA measurements.

Histological assessment

Mice were killed after an overdose of isoflurane anaesthesia and the inflamed hind knees were surgically removed. The knees were fixed in 4% paraformaldehyde (PFD) for 48 h, decalcified in 0.5 M EDTA for 1 week, and then kept in 30% sucrose until they were cut into 10 μm sections. Tissue sections were stained with haematoxylin and eosin (Electron Microscopy Sciences , Hatfield, PA, USA). From each knee joint, three sections were taken at different depths. Histopathological changes in the knee joints were measured using a semi-quantitative scoring system to assess inflammation, synovial hyperplasia, and cartilage and bone erosion as previously described (0–4 scale for each feature; maximum score of 12 per mouse) (Luo *et al.*, 2011; Ahmed *et al.*, 2012).

Assessment of evoked pain (thermal and mechanical sensitivity)

Thermal sensitivity was measured using the Hargreaves' method as previously described (Gao *et al.*, 2013). The baseline latencies were set to approximately 10 s with a maximum of 20 s as the cut-off to prevent potential injury. The latencies were averaged over three trials, separated by 30 min intervals. Mechanical sensitivity was measured using a series of von Frey filaments (North Coast Medical, Inc., Gilroy, CA, USA) as previously described (Gao *et al.*, 2013). The smallest monofilament that evoked paw withdrawal responses on three out of five trials was taken as the mechanical threshold. Pain sensitivity was assessed before immunization to establish a baseline level, before the onset of arthritis (21 and 28 days after immunization), and up to 10 days after the onset of arthritis (inflamed hind paws were assessed).

Assessment of spontaneous behaviour

The spontaneous activity of mice was tested using an open-field system (Med Associates, Inc., St. Albans, VT, USA), which automatically detects the free movements of mice. Activity monitor software was used to record and quantify behaviours, including the distance travelled and vertical time. Animals were acclimatized to the equipment 30 min before the test. The spontaneous activity of mice was tested on day 36 in the pretreatment experiment. In the treatment experiment, activity was tested before the onset of arthritis to establish the baseline, and on days 0, 5 and 10 after onset. All tests were conducted for 30 min.

Measurement of TNF-α, IL-1β and IL-6 in the paw

Mice were anaesthetized with isoflurane, and the inflamed paws were removed. The entire paw was weighed and homogenized using a tissuemiser (Thermo Fisher Scientific, Pittsburgh, PA, USA) with three volumes of buffer containing 50 mM NaCl, 10 mM Tris, 2.5 mM $MgCl_2$, 0.1 mM PMSF, and protease inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL, USA), with pH adjusted to 7.4. The tissue samples were centrifuged at 15 000× *g* for 10 min. Tissue supernatants were collected for the assay. TNF-α, IL-1β and IL-6 concentrations were measured by ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Measurement of phosphorylated ERK, p38, JNK and calmodulin-dependent PKII (CaMKIIα) proteins levels in the spinal cord

Mice were killed after an overdose of isoflurane anaesthesia. The lumbar section of the spinal cord was dissected and homogenized using a Dounce homogenizer in an ice-cold RIPA buffer containing 50 mM Tris HCl, 150 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, 2% SDS, 1% deoxycholate, 0.1 mM PMSF, and protease inhibitor cocktails (Thermo Fisher Scientific). The lysed tissues were sonicated at a

constant intensity of 2.5 for 10 s, and centrifuged at 18 000× *g* (4°C) for 5 min. The concentrations of total protein were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. Protein samples were heated at 100°C for 5 min, electrophoresed in 10% SDS polyacrylamide gel, and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The blots were blocked with 5% skimmed milk in Tris-buffered saline–Tween 0.1% for 1 h at room temperature and probed with rabbit anti-p-ERK (1:1000, Cell Signal, Danvers, MA, USA), anti-ERK (1:10000, Cell Signal), anti-p-p38 (1:1000, Cell Signal), anti-p38 (1:5000, Cell Signal), anti-p-CaMKIIα (1:1000, Cell Signal), anti-CaMKIIα (1:1000, Cell Signal), anti-p-JNK (1:1000, Cell Signal), anti-JNK (1:5000, Cell Signal) and anti-GAPDH (1:10 000, ProSci, Inc., Poway, CA, USA) primary antibodies at 4°C overnight. The blots were washed and incubated for 1 h at room temperature with the HRP-conjugated secondary antibody (1:10 000, Cell Signal), then developed with enhanced chemiluminescence (Millipore). The densitometry of protein bands was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Drug treatment

YM-58483 was purchased from Tocris (Minneapolis, MN, USA). YM-58483 was dissolved in DMSO as a stock solution and further diluted to final concentrations in 1% DMSO/cremophor EL with saline for oral administration. For pretreatment experiments, drugs were given from days 28 to 37 after the first collagen injection. For treatment experiments, drugs were given for 10 days, beginning from the day of onset.

Data analysis

Data are presented as mean ± SEM. Treatment effects were statistically analysed with a one-way ANOVA. Pair-wise comparisons between means were tested using the *post hoc* Bonferroni method. Error probabilities of $P < 0.05$ were considered statistically significant. The Origin 8.1 software (OriginLab Corp., Northampton, MA, USA) was used to perform all statistical analyses.

Results

Pretreatment with YM-58483 prevents the development of the symptoms of CIA

To determine whether SOC channels play a role in CIA, we examined the effect of YM-58483, a potent SOC channel inhibitor, on CIA in a mouse model that shares many hallmarks with human RA. DBA1 mice were injected with collagen on days 1 and 21. On day 27, collagen-treated mice were divided into three treatment groups: vehicle control, YM-58483 5 mg·kg^{-1} and 10 mg·kg^{-1}. YM-58483 or vehicle was administered daily from days 28 to 37. Mice started developing CIA 28 days after first injection of collagen, with small, but detectable increases in paw volume observed by day 28 and increases in clinical severity score observed by day 32 (Figure 1A). The incidence of CIA in the control group was 70% and oral administration of YM-58483 from days 28 to 37 significantly decreased the incidence of CIA, with 29 and 21% in 5 and 10 mg·kg^{-1} groups separately. Specifically, pretreatment with YM-58483 completely prevented the increase in clinical score observed in vehicle-treated mice and also significantly inhibited the development of paw oedema (Figure 1A), suggesting YM-58483 prevented the development of collagen-induced inflammation.

Treatment with YM-58483 reduces the symptoms of CIA

To determine whether YM-58483 can reverse or prevent the worsening of inflammation and other signs and features of arthritis disease, YM-58483 was administered for 10 days after the onset of CIA. By the end of the 10 day treatment period, the 10 mg·kg^{-1} dose of YM-58483 had significantly reduced the clinical severity score, while the 5 mg·kg^{-1} dose prevented the increase in clinic score observed in the vehicle-treated control group (Figure 1B). Similarly, the higher dose of YM-58483 significantly reduced the paw oedema while the lower dose prevented the increase seen in the vehicle-treated control group (Figure 1B). These results clearly demonstrate that YM-58483 has therapeutic effects in this model, suggesting an involvement of SOC channels in CIA.

YM-58483 reduces joint destruction in CIA mice

RA is characterized by the presence of an inflammatory synovitis accompanied by destruction of joint cartilage and bone (Gravallese, 2002). In the present study, knee joint histology was assessed at the end of the treatment by haematoxylin and eosin staining. Joints from PBS-injected normal mice showed no signs of histopathological changes with a histological score of zero. CIA control mice showed pronounced infiltration of inflammatory cells, synovial hyperplasia, and erosion of articular cartilage compared with the PBS-injected mice. YM-58483 dose-dependently reduced synovial inflammation, synovial hyperplasia and bone erosions and overall histological score (Figure 2). These results suggest that YM-58483 can effectively inhibit the joint inflammation associated with CIA.

YM-58483 attenuates thermal and mechanical hypersensitivity in CIA mice

Pain is one of the first and most disabling manifestations in patients with arthritis (Zatarain and Strand, 2006). Our previous study demonstrated that YM-58483 has strong analgesic effects (Gao *et al.*, 2013), so we wanted to determine whether these analgesic effects extended to arthritic pain. CIA mice developed mechanical allodynia on day 21 and thermal hyperalgesia on day 28 after the first injection of collagen (Figure 3A); prior to the onset of most other symptoms of CIA. Both mechanical allodynia and thermal hyperalgesia reached their peaks when CIA developed by day 32 and remained at peak levels for at least 10 days. YM-58483 dose-dependently reduced mechanical allodynia and prevented the development of thermal hyperagesia when administered before the onset of CIA (Figure 3A). Importantly, the 10 mg·kg^{-1} dose of YM-58483 significantly attenuated mechanical allodynia, and completely reversed thermal hyperalgesia when administered after pain was fully

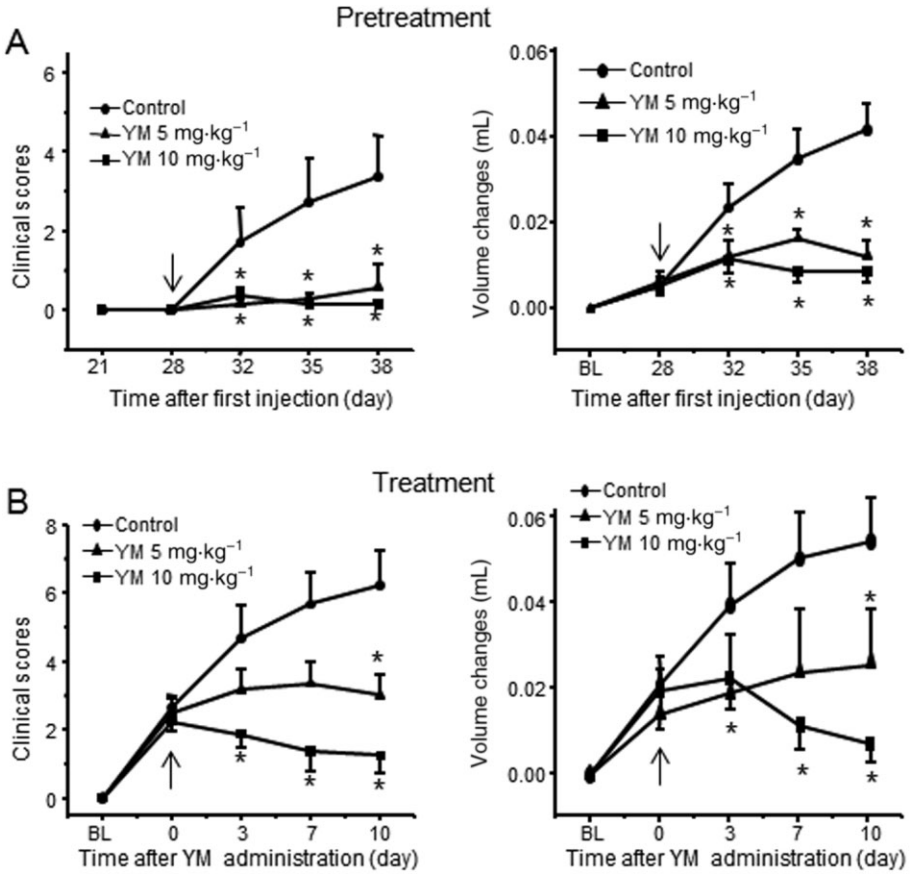

Figure 1

Effect of YM-58483 on clinical score and paw oedema in CIA mice. (A) Effect of pretreatment. YM-58483 or vehicle was administered daily from days 28 to 37. Pretreatment with YM-58483 prevented the development of clinical signs (left panel) and significantly reduced the magnitude of paw oedema (right panel) in CIA mice ($n = 7$–11). (B) Effect of treatment. YM-58483 or vehicle was administered for 10 days after the development of symptoms. Treatment with YM-58483 resulted in a dose-dependent reduction in clinical signs and paw oedema ($n = 6$–9). Arrows indicate when the drug was first administered. Values represent mean ± SEM; *$P < 0.05$, compared with control by one-way ANOVA.

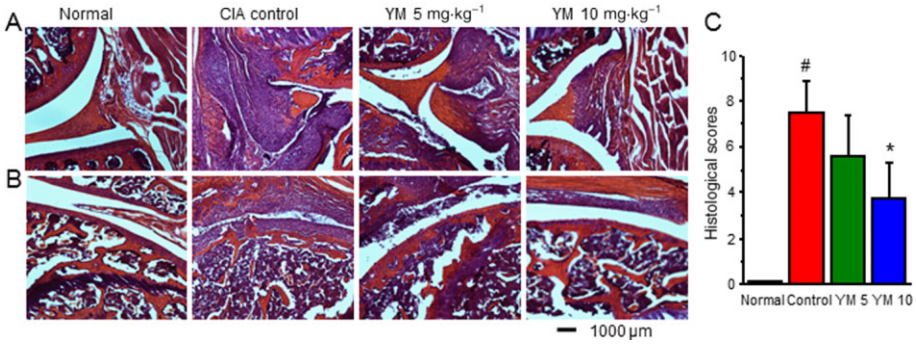

Figure 2

Effect of treatment with YM-58483 on knee joint histology. (A and B) Representative photomicrographs of haematoxylin and eosin-stained sections of synovium (A) and cartilage (B) from knee joints obtained after 10 days of drug treatment. Magnification is 10× and scale bar = 1000 μm. (C) Combined histological score of inflammation, hyperplasia and erosion. Treatment with 5 or 10 mg·kg^{-1} YM-58483 resulted in a dose-dependent reduction in the histological score in CIA mice ($n = 6$–9). Values represent mean ± SEM; #$P < 0.05$, compared with normal by Student's t-test; *$P < 0.05$, compared with control by one-way ANOVA.

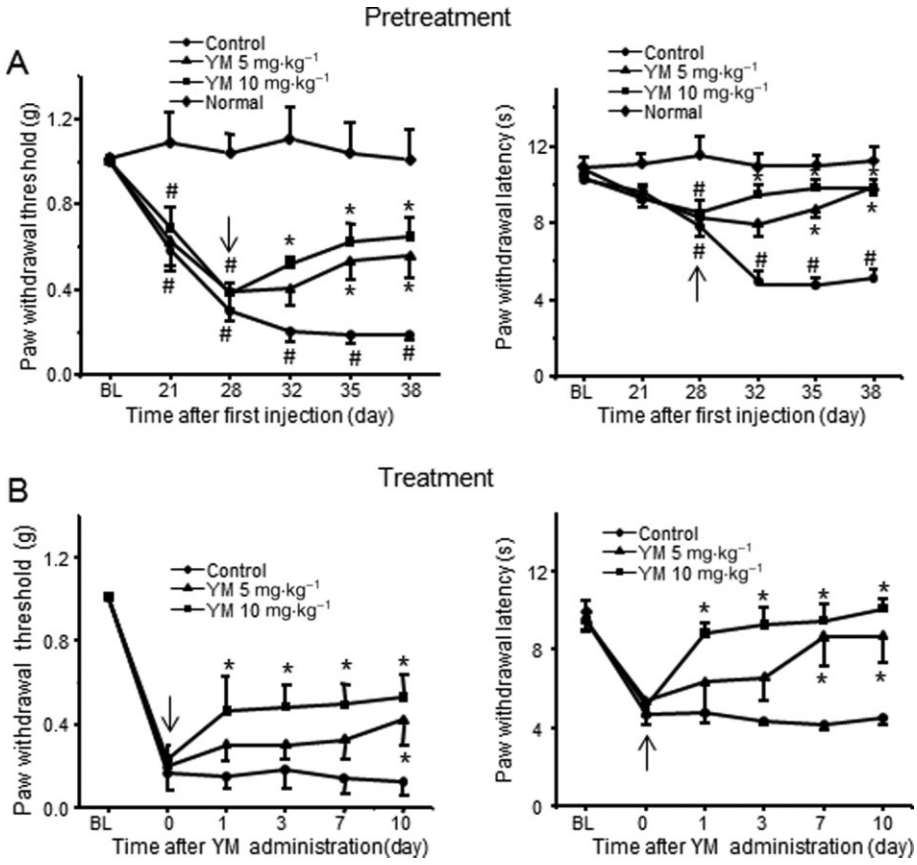

Figure 3

Effect of YM-58483 on CIA-induced thermal and mechanical hypersensitivity. (A) Effects of pretreatment. Administration of YM-58483 resulted in a dose-dependent attenuation of mechanical allodynia (left panel) and thermal hyperalgesia (right panel) in CIA mice (n = 7–11). (B) Effects of treatment. YM-58483 produced a dose-dependent reversal of mechanical allodynia (left panel) and thermal hyperalgesia (right panel) (n = 6–9). Arrows indicate when the drug was first administered. Values represent mean ± SEM; #P < 0.05, compared with normal by one-way ANOVA; *P < 0.05, compared with control by one-way ANOVA.

established (Figure 3B). These results suggest that YM-58483 produces a strong analgesic effect in arthritic pain.

YM-58483 improves deficits in spontaneous movement in CIA mice

Arthritic joint pain can be present at rest, but typically worsens with activity or movement of the affected joint, leading to disability and reduction of the patients' quality of life (Cheng and Penninger, 2004). To assess the effect of YM-58483 on deficits in spontaneous movement associated with CIA, we tested spontaneous activity in mice using the open-field system. Total travel distance and vertical movement were significantly reduced in CIA mice compared with normal mice that were not treated with collagen when tested on day 36. Pretreatment of CIA mice with 5 or 10 mg·kg^{-1} YM-58483 completely prevented the reduction in spontaneous activities (Figure 4A). Treatment of CIA mice with 5 or 10 mg·kg^{-1} YM-58483 partially reversed the deficits in both total distance and vertical time on day 5. Although there was also a trend for reversal on day 10, the effects were not significant because of an apparent recovery in the untreated CIA mice (Figure 4B). These results suggest that YM-58483 improves deficits in spontaneous movement in CIA mice.

YM-58483 decreases production of cytokines in inflamed paw of CIA mice

Various pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are present in the joints of RA patients and numerous studies have provided evidence that these pro-inflammatory cytokines play an important role in the development of arthritis and arthritis-induced pain (Constandil *et al.*, 2009; Segond von Banchet *et al.*, 2009). We have previously shown that YM-58483 inhibits the production of TNF-α and IL-1β in mice with CFA-induced inflammation of the paw (Gao *et al.*, 2013). To better understand the peripheral mechanisms that contribute to the ability of YM-58483 to prevent or reduce the effects of CIA, we measured levels of TNFα, IL-1β and IL-6 in the paws of normal and CIA mice. Production of IL-1β, IL-6 and TNF-α was dramatically increased in the inflamed paw of CIA mice and both pretreatment and treatment with YM-58483 dose-dependently

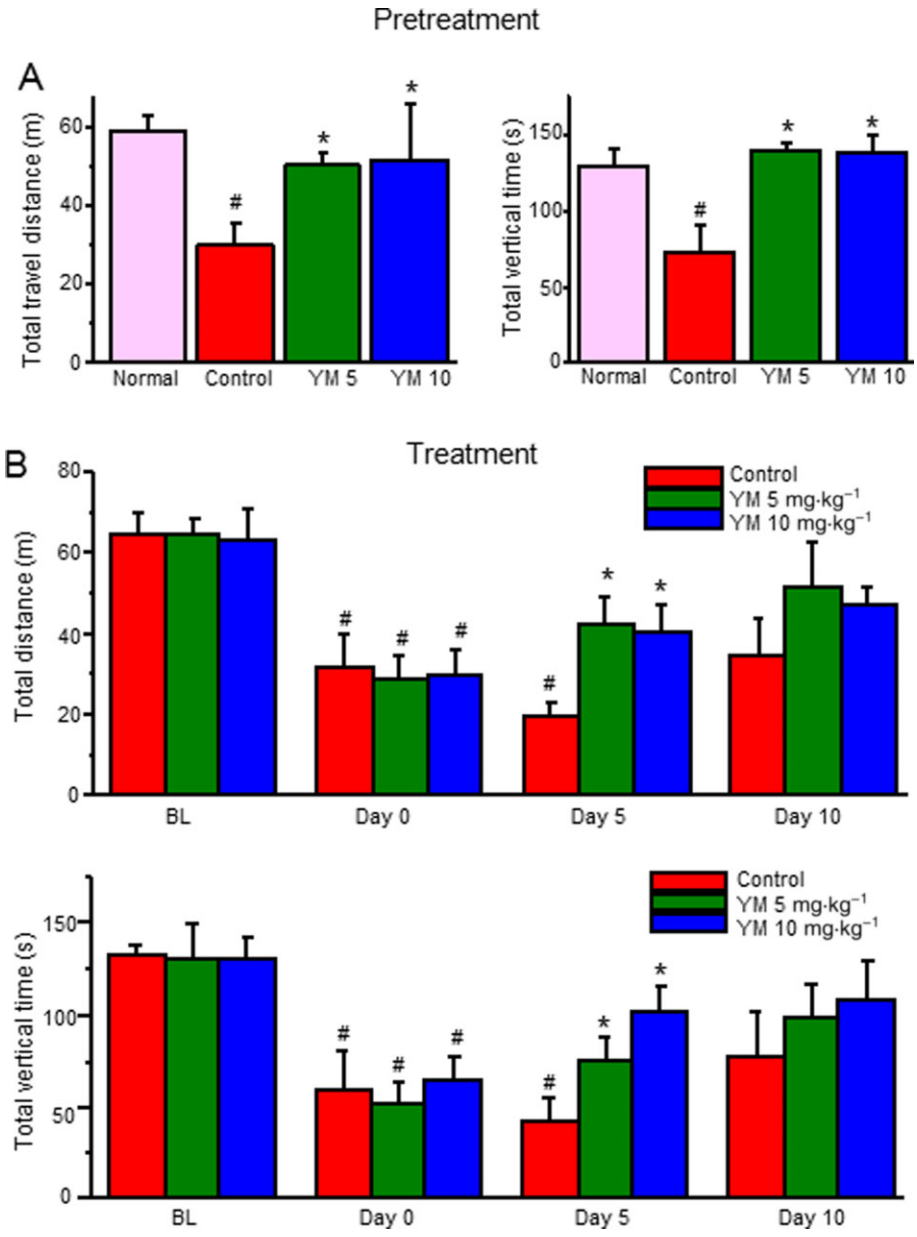

Figure 4

Effect of YM-58483 on spontaneous activity in CIA mice. (A) Effects of pretreatment. Administration of 5 or 10 mg·kg^{-1} YM completely prevented the CIA-induced reduction in distance travelled and time performing vertical movements (n = 7–11). (B) Effects of treatment. Administration of 5 or 10 mg·kg^{-1} YM-58483 produced a significant increase in both total distance and total time spent performing vertical movements on day 5, but had no effect on these endpoints on day 10 because of an apparent trend towards recovery in the CIA control group (n = 6–9). Values represent mean ± SEM; #P < 0.05, compared with normal or baseline by one-way ANOVA; *P < 0.05, compared with control by one-way ANOVA.

decreased these pro-inflammatory cytokine levels (Figure 5). These results strongly suggest that the therapeutic effects of YM-58483 in CIA mice are associated with reductions in pro-inflammatory cytokine production.

YM-58483 reduces activation of ERK and CaMKIIα in the spinal cord

There is increasing recognition that peripheral inflammation cannot fully explain the level of pain in RA and central sensitization at the spinal level is also thought to play a role in arthritic pain (Christianson *et al.*, 2010; Goldenberg *et al.*, 2011). It is well-documented that the MAPKs and CaMKIIα signal pathways contribute to central sensitization (Sheng and Kim, 2002; Lee *et al.*, 2011). To determine whether MAPK and CaMKIIα pathways at spinal level are activated in the CIA model and whether they are modulated by YM-58483, the expression levels of p-ERK, p-p38, p-JNK and p-CaMKIIα in the lumbar segments of the spinal cord were evaluated by Western blot analysis in the treatment experiment 10 days after onset of CIA. p-ERK and p-p38, but not p-JNK, were

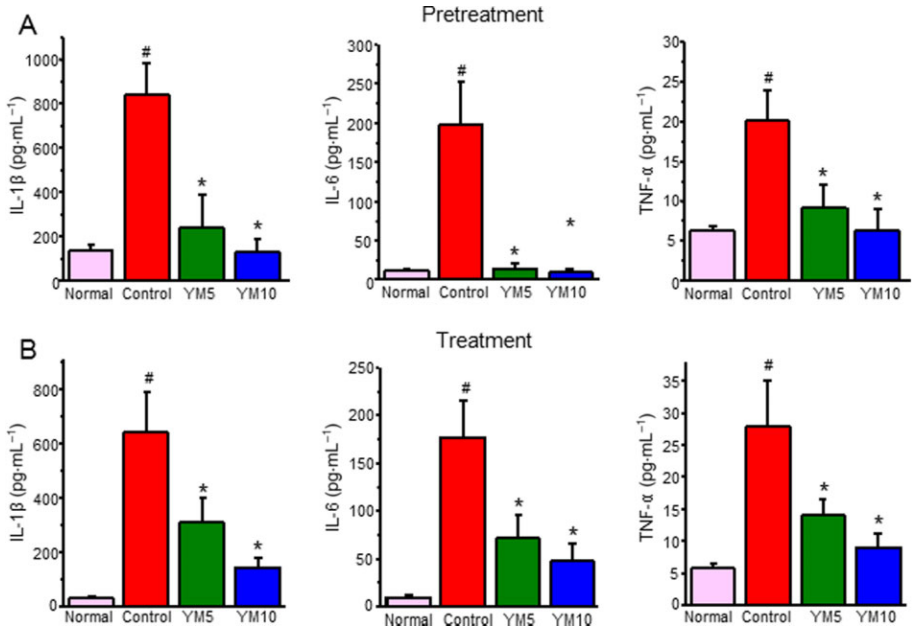

Figure 5

Effect of YM-58483 on cytokine release in the paws of CIA mice. (A) Effect of pretreatment. Administration of 5 or 10 mg·kg^{-1} YM-58483 completely prevented the increase in IL-1β, IL-6 and TNF-α release observed in CIA control mice (n = 7–11). (B) Effect of treatment. Administration of 5 or 10 mg·kg^{-1} YM-58483 resulted in a dose-dependent reversal of the increase in IL-1β, IL-6 and TNF-α release observed in CIA control mice (n = 6–9). Values represent mean ± SEM. #P < 0.05, compared with normal by Student's t-test; *P < 0.05, compared with control by one-way ANOVA.

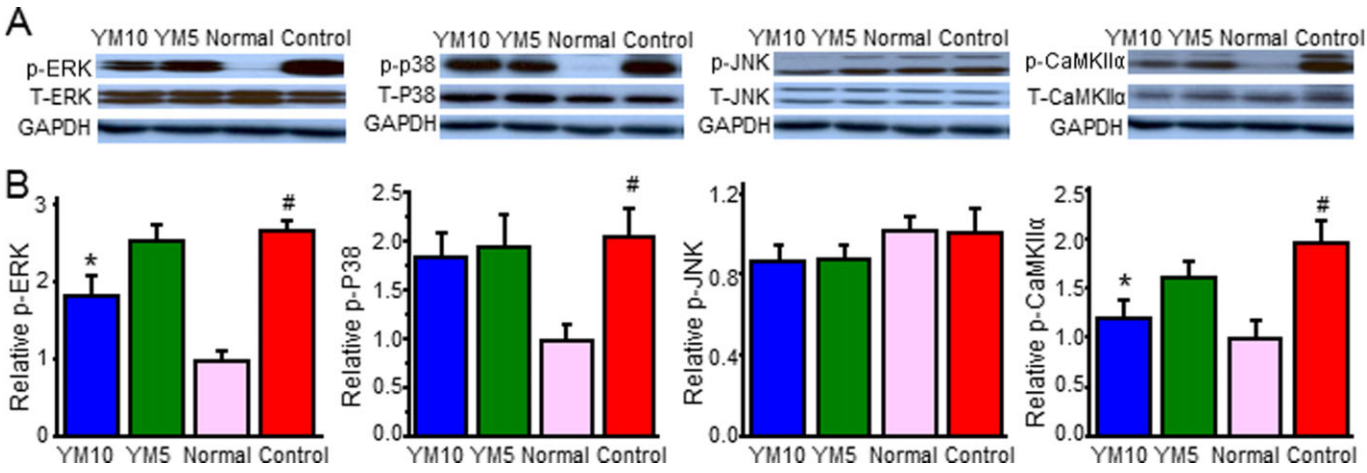

Figure 6

Effect of YM-58483 on MAPK and CaMKIIα signalling in the spinal cord of CIA mice. (A) Representative immunoblots of p-ERK, p-p38, p-JNK and p-CaMKIIα in spinal cord samples. (B) Administration of 5 or 10 mg·kg^{-1} YM-58483 for 10 days after onset of CIA symptoms resulted in a dose-dependent reversal of increases in p-ERK and p-CaMKIIα, but had no effect on increases in p-p38. The densities of the p-ERK, p-p38, p-JNK and p-CaMKIIα bands were normalized to total (T) ERK, p38, JNK or CaMKIIα bands respectively. Values represent mean ± SEM. n = 6–9. #P < 0.05, compared with normal group by Student's t-test; *P < 0.05, compared with CIA control group by one-way ANOVA.

significantly increased in the spinal cord of CIA mice compared with normal mice. Treatment with 10 mg·kg^{-1} YM-58483 after the onset of CIA significantly reduced the activation of p-ERK, but not p-p38 in the CIA mice (Figure 6). The level of p-CaMKIIα was also significantly increased in CIA mice compared with normal controls and treatment with 10 mg·kg^{-1} YM-58483 significantly reduced the activation of CaMKIIα (Figure 6). These correlative findings suggest that YM-58483-induced analgesic action may also be mediated by modulation of central ERK and CaMKIIα pathways.

Discussion

The present study demonstrates that the SOC channel inhibitor YM-58483 is able to effectively prevent or reverse histopathological, biochemical, pain-related behavioural and other symptoms of CIA mice. Treatment with YM-58483 reduces or prevents collagen-induced inflammation in the paw, inflammation and erosion in the knee joint, attenuates mechanical and thermal hypersensitivity, and improves deficits in spontaneous movement in CIA mice. This study provides the first *in vivo* evidence that inhibition of SOC channels produces robust anti-inflammatory and analgesic actions in CIA mice. A recent *in vitro* study reported that both the expression and function of Orai1 are upregulated in the naïve CD4(+) T-cells from RA patients, which is associated with cytokine release (Liu *et al.*, 2014), further supporting the prospect that SOC channels are potential drug targets for the treatment of RA.

RA is characterized by synovial inflammation, erosion of cartilage and bone associated with autoreactive T- and B-cells. SOC channels have been implicated as critical players in immune and inflammatory diseases (Feske, 2009; Parekh, 2010) and multiple studies have established the critical role of SOCE in the functions of T-cells and B-cells (Hogan *et al.*, 2003; McCarl *et al.*, 2010). Consequently, it has been suggested that SOC channels may play an important role in RA (Lin *et al.*, 2013; Liu *et al.*, 2014). However, there is no direct *in vivo* evidence to support this notion. Our previous study demonstrated that YM-58483 strongly reduces CFA-induced inflammation (Gao *et al.*, 2013) and we hypothesized that YM-58483 would also reduce inflammation in CIA mice. Consistent with this hypothesis, we found that treatment with YM-58483 significantly decreased joint inflammation in CIA mice by reducing synovial inflammation, synovial hyperplasia and bone erosion. Moreover, pretreatment with YM-58483 markedly reduced the incidence of CIA and dramatically decreased the clinical score and paw oedema of collagen-treated mice in both pretreatment and treatment experiments. These findings indicate that SOC channels may play an important role in arthritis. Although the cause of RA is unknown, it is well established that pro-inflammatory cytokines play a crucial role in RA. The pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are involved in each phase of the pathogenesis of RA, promoting autoimmunity, maintaining chronic inflammatory synovitis and driving the destruction of adjacent joint tissue (McInnes and Schett, 2007). Evidence from clinical studies and animal work supports the view that disruption of pro-inflammatory cytokine action with biotherapeutics is an effective strategy for the treatment of RA (Nixon *et al.*, 2007; Navarro-Millan *et al.*, 2012). Pro-inflammatory cytokines also contribute to the generation of inflammatory pain and peripheral pain sensitization (Woolf *et al.*, 1997). In agreement with a previous study (Moon *et al.*, 2014), our ELISA results show that IL-1β, IL-6 and TNF-α levels are dramatically increased in the inflamed paw of CIA mice. Treatment with YM-58483 attenuates production of these cytokines in a dose-dependent manner. It is likely that a reduction in peripheral pro-inflammatory cytokine production is one of the mechanisms underlying the effect of YM-58483 on CIA and arthritic pain.

Pain, is a very common symptom in RA, affects patients' quality of life and generally represents the first sign of the disease and the primary reason for seeking treatment. Diminishing pain appeared as the main outcome of treatment for 81% of RA patients (Cunha-Miranda *et al.*, 2010). However, strategies to effectively and specifically alleviate arthritic pain are very limited. One reason for the paucity of effective treatments is that arthritic pain is a condition that involves a complex pathological interaction of central sensitization and peripheral inflammation. An ideal strategy should target both peripheral inflammation and central sensitization. We have previously shown that YM-58483 not only reduces peripheral inflammation, but also produces a central analgesic effect in chronic pain models (Gao *et al.*, 2013). In the present study, we also found that YM-58483 attenuates mechanical allodynia and thermal hyperalgesia of the hind paw in CIA mice. Arthritic pain typically worsens with weight bearing and activity or movement of the affected joint. The activity of rodents has been used as a good surrogate for joint pain measurements in arthritis (Inglis *et al.*, 2007). In this study, CIA mice showed large reductions in distance travelled and vertical movement and treatment with YM-58483 significantly improved the deficits in these activities. The potent analgesic effect of YM-58483 makes the role of SOC channels in CIA more meaningful as it reduces both CIA and arthritic pain at the same time, suggesting that SOC channels may be ideal targets for RA treatments. Inhibition of SOC channels in the CIA model resulted in dramatic disease-modifying and symptom-alleviating effects.

Arthritic pain is traditionally thought to be mediated by the activation of immune cells and their mediators. However, conventional RA pain management strategies are not very effective. In a cross-sectional analysis of 12 090 RA patients recruited from rheumatology practices, pain levels were almost constant over RA duration, even though most participants were treated with a disease-modifying anti-rheumatic drugs, an anti-TNF-α agent, or both (Wolfe *et al.*, 2010). Increasing evidence suggests that central sensitization contributes to the persistent pain in this disorder. It is well known that MAPK and CaMKIIα pathways are involved in central sensitization. Numerous studies have demonstrated that ERK1/2, p38 and JNK at the spinal level are activated in inflammatory pain, including monoarthritis (Cruz *et al.*, 2005), adjuvant-induced arthritis (Wada *et al.*, 2005) and collagen antibody-induced arthritis (CAIA) (Bas *et al.*, 2012), but little is known about CIA. It has also been shown that SOCE is involved in ERK and CaMKIIα activation in non-neuronal cells (Voelkers *et al.*, 2010). It is reasonable to hypothesize that SOC channels are involved in central sensitization. In the present study, we discovered that ERK1/2 and p38, but not JNK, are activated in the spinal cord after 10 days of onset, suggesting ERK and p38 MAPK pathways are involved in arthritic pain. A previous study has shown that JNK is activated on day 26 (late phase), but not on day 10 (first phase) after the onset of CAIA model (Bas *et al.*, 2012). It is possible that JNK is activated at the late stage in the CIA model. YM-58483 10 $mg \cdot kg^{-1}$ significantly reduced ERK, but not p38 activation. Activation of p38 in the spinal cord appears localized to microglia (Svensson *et al.*, 2003), suggesting that the YM-58483-induced analgesic effect is not mediated by p38 activation in microglia. The role of CaMKIIα in

central sensitization has also been well accepted. Numerous studies have reported the activation of CaMKIIα in several pain models and the inhibition of CaMKIIα attenuates pain involving central sensitization (Fang *et al.*, 2002; Crown *et al.*, 2012). The role of CaMKIIα in arthritic pain associated with CIA has not been established yet. Our results reveal that CaMKIIα is activated in the spinal cord after 10 days of onset and YM-58483 dose-dependently inhibited the CaMKIIα activation, suggesting CaMKIIα activation plays a role in arthritic pain. Taken together, our findings indicate that spinal SOC channels are involved in central signal transduction pathways.

It has been proposed that three mechanisms of YM-58483 account for its inhibitory effects on Ca^{2+} influx in non-excitable cells: the inhibition of SOCs in immune cells, such as T-lymphocytes, mast cell and platelets (Zitt *et al.*, 2004; Harper and Poole, 2011; Law *et al.*, 2011), the inhibition of the TRPC channels in HEK293 cells (He *et al.*, 2005), and the facilitation of TRPM4 in Jurkat cells (Takezawa *et al.*, 2006). A previous study has shown that Ca^{2+} entering cells through calcium release-activated calcium (CRAC) channels can activate TRPM4, leading to depolarization and reducing the driving force for Ca^{2+} entry through CRAC channels (Launay *et al.*, 2004). TRPM4 channels are expressed widely in peripheral tissues and sparsely in the brain (Launay *et al.*, 2002; Fonfria *et al.*, 2006); they have not been reported to be expressed in dorsal horn neurons. We did not observe YM-induced currents at 0.3 and 3.0 μM concentrations (data not shown). In contrast, SOC channel activation-induced depolarization of neurons was blocked, not enhanced by YM-58483 (Xia *et al.*, 2014), suggesting that the TRPM4 channel is not functional in dorsal horn neurons. If TRPM4 is functionally expressed in dorsal horn neurons, activation of TRPM4 by YM-58483 would cause depolarization of neurons and lead to neuronal firing, which could result in more pain. In fact, YM-58483 drastically attenuated pain (Gao *et al.*, 2013). Therefore, the YM-58483-induced analgesic effect is unlikely to be mediated by activation of TRPM4. In peripheral T-lymphocytes and mast cells, YM-58483 inhibits cytokine production with similar potency as found for inhibition of SOCE (Zitt *et al.*, 2004; Law *et al.*, 2011), suggesting the YM-induced peripheral effect is likely to be mediated by inhibition of SOCE. A previous report suggested that YM-58483 activated TRPM4-like channels in Jurkat cells (Takezawa *et al.*, 2006). However, the role of TRPM4 in cytokine production in native immune cells has not been established (Weber *et al.*, 2010). We and others have shown that YM-58483 is very potent for SOCE in both dorsal horn neurons and immune cells (Zitt *et al.*, 2004; Law *et al.*, 2011; Chen *et al.*, 2013; Xia *et al.*, 2014), suggesting that YM-58483-induced anti-CIA is possibly mediated by inhibition of SOCE. However, it has been shown that YM-58483 blocks TRPC channels, which are components of SOC channels in several cell types (Ong *et al.*, 2007; Alkhani *et al.*, 2014). We do not rule out the possibility that TRPC channels are involved in YM-58483-induced anti-CIA.

In summary, in the present study it was demonstrated that the SOC channel inhibitor YM-58483 not only prevents the development of CIA, but also produces strong anti-inflammatory and analgesic actions in established CIA. The results of the mechanistic studies suggest that these actions were mediated by reducing peripheral cytokines and decreasing ERK and CaMKIIα activation in the spinal cord. It has been demonstrated that inhibition of SOCE by YM-58483 prevents antigen-induced T-cell responses (Ohga *et al.*, 2008a). Our findings provide further direct evidence that targeting SOC channels has the exciting potential to lead to a new approach for the prevention and treatment of RA and the pain associated with RA.

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Author contributions

H. H., X. H. G. and Y. D. conceived of the project and designed experiments. X. H. G., R. G., Y. Z. T. and H. H. performed all experiments, analysed data. X. H. G., H. H., P. M., J. E. B. and R. G. prepared the paper. All authors read and approved the paper.

Conflict of interest

None declared.

References

Ahmed AS, Li J, Erlandsson-Harris H, Stark A, Bakalkin G, Ahmed M (2012). Suppression of pain and joint destruction by inhibition of the proteasome system in experimental osteoarthritis. Pain 153: 18–26.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Ion channels. Br J Pharmacol 170: 1607–1651.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Alkhani H, Ase AR, Grant R, O'Donnell D, Groschner K, Seguela P (2014). Contribution of TRPC3 to store-operated calcium entry and inflammatory transductions in primary nociceptors. Mol Pain 10: 43.

Bas DB, Su J, Sandor K, Agalave NM, Lundberg J, Codeluppi S *et al.* (2012). Collagen antibody-induced arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency. Arthritis Rheum 64: 3886–3896.

Chen G, Panicker S, Lau KY, Apparsundaram S, Patel VA, Chen SL *et al.* (2013). Characterization of a novel CRAC inhibitor that potently blocks human T cell activation and effector functions. Mol Immunol 54: 355–367.

Cheng HY, Penninger JM (2004). DREAMing about arthritic pain. Ann Rheum Dis 63 (Suppl. 2): ii72–ii75.

Christianson CA, Corr M, Firestein GS, Mobargha A, Yaksh TL, Svensson CI (2010). Characterization of the acute and persistent pain state present in K/BxN serum transfer arthritis. Pain 151: 394–403.

Constandil L, Hernandez A, Pelissier T, Arriagada O, Espinoza K, Burgos H *et al.* (2009). Effect of interleukin-1beta on spinal cord nociceptive transmission of normal and monoarthritic rats after disruption of glial function. Arthritis research & therapy 11: R105.

Crown ED, Gwak YS, Ye Z, Yu Tan H, Johnson KM, Xu GY *et al.* (2012). Calcium/calmodulin dependent kinase II contributes to persistent central neuropathic pain following spinal cord injury. Pain 153: 710–721.

Cruz CD, Neto FL, Castro-Lopes J, McMahon SB, Cruz F (2005). Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats. Pain 116: 411–419.

Cunha-Miranda L, Costa L, Ribeiro JS (2010). NEAR study: Needs and Expectations in Rheumatoid ARthritis – do we know our patients needs? Acta Reumatol Port 35: 314–323.

Di Sabatino A, Rovedatti L, Kaur R, Spencer JP, Brown JT, Morisset VD *et al.* (2009). Targeting gut T cell Ca2+ release-activated Ca2+ channels inhibits T cell cytokine production and T-box transcription factor T-bet in inflammatory bowel disease. J Immunol 183: 3454–3462.

Fang L, Wu J, Lin Q, Willis WD (2002). Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J Neurosci 22: 4196–4204.

Feske S (2009). ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca2+ entry in the immune system and beyond. Immunol Rev 231: 189–209.

Fonfria E, Murdock PR, Cusdin FS, Benham CD, Kelsell RE, McNulty S (2006). Tissue distribution profiles of the human TRPM cation channel family. J Recept Signal Transduct Res 26: 159–178.

Gao R, Gao X, Xia J, Tian Y, Barrett JE, Dai Y *et al.* (2013). Potent analgesic effects of a store-operated calcium channel inhibitor. Pain 154: 2034–2044.

Gerecz-Simon EM, Tunks ER, Heale JA, Kean WF, Buchanan WW (1989). Measurement of pain threshold in patients with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and healthy controls. Clin Rheumatol 8: 467–474.

Goldenberg DL, Clauw DJ, Fitzcharles MA (2011). New concepts in pain research and pain management of the rheumatic diseases. Semin Arthritis Rheum 41: 319–334.

Gravallese EM (2002). Bone destruction in arthritis. Ann Rheum Dis 61 (Suppl. 2): ii84–ii86.

Harper MT, Poole AW (2011). Store-operated calcium entry and non-capacitative calcium entry have distinct roles in thrombin-induced calcium signalling in human platelets. Cell Calcium 50: 351–358.

He LP, Hewavitharana T, Soboloff J, Spassova MA, Gill DL (2005). A functional link between store-operated and TRPC channels revealed by the 3,5-bis(trifluoromethyl)pyrazole derivative, BTP2. J Biol Chem 280: 10997–11006.

Hogan PG, Chen L, Nardone J, Rao A (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. Genes & development 17: 2205–2232.

Inglis JJ, Notley CA, Essex D, Wilson AW, Feldmann M, Anand P *et al.* (2007). Collagen-induced arthritis as a model of hyperalgesia: functional and cellular analysis of the analgesic actions of tumor necrosis factor blockade. Arthritis Rheum 56: 4015–4023.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Latremoliere A, Woolf CJ (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 10: 895–926.

Launay P, Fleig A, Perraud AL, Scharenberg AM, Penner R, Kinet JP (2002). TRPM4 is a Ca2+-activated nonselective cation channel mediating cell membrane depolarization. Cell 109: 397–407.

Launay P, Cheng H, Srivatsan S, Penner R, Fleig A, Kinet JP (2004). TRPM4 regulates calcium oscillations after T cell activation. Science 306: 1374–1377.

Law M, Morales JL, Mottram LF, Iyer A, Peterson BR, August A (2011). Structural requirements for the inhibition of calcium mobilization and mast cell activation by the pyrazole derivative BTP2. Int J Biochem Cell Biol 43: 1228–1239.

Lee Y, Pai M, Brederson JD, Wilcox D, Hsieh G, Jarvis MF *et al.* (2011). Monosodium iodoacetate-induced joint pain is associated with increased phosphorylation of mitogen activated protein kinases in the rat spinal cord. Mol Pain 7: 39.

Lee YC (2013). Effect and treatment of chronic pain in inflammatory arthritis. Curr Rheumatol Rep 15: 300.

Lewis RS (2007). The molecular choreography of a store-operated calcium channel. Nature 446: 284–287.

Lin FF, Elliott R, Colombero A, Gaida K, Kelley L, Moksa A *et al.* (2013). Generation and characterization of fully human monoclonal antibodies against human Orai1 for autoimmune disease. J Pharmacol Exp Ther 345: 225–238.

Liu S, Watanabe S, Shudou M, Kuno M, Miura H, Maeyama K (2014). Upregulation of store-operated Ca(2+) entry in the naive CD4(+) T cells with aberrant cytokine releasing in active rheumatoid arthritis. Immunol Cell Biol 92: 752–760.

Luo Y, Boyle DL, Hammaker D, Edgar M, Franzoso G, Firestein GS (2011). Suppression of collagen-induced arthritis in growth arrest and DNA damage-inducible protein 45beta-deficient mice. Arthritis Rheum 63: 2949–2955.

Ma J, McCarl CA, Khalil S, Luthy K, Feske S (2010). T-cell-specific deletion of STIM1 and STIM2 protects mice from EAE by impairing the effector functions of Th1 and Th17 cells. Eur J Immunol 40: 3028–3042.

McCarl CA, Khalil S, Ma J, Oh-hora M, Yamashita M, Roether J *et al.* (2010). Store-operated Ca2+ entry through ORAI1 is critical for T cell-mediated autoimmunity and allograft rejection. J Immunol 185: 5845–5858.

McInnes IB, Schett G (2007). Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 7: 429–442.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Meeus M, Vervisch S, De Clerck LS, Moorkens G, Hans G, Nijs J (2012). Central sensitization in patients with rheumatoid arthritis: a systematic literature review. Semin Arthritis Rheum 41: 556–567.

Moon YM, Lee J, Lee SY, Her YM, Ryu JG, Kim EK *et al.* (2014). Gene associated with retinoid-interferon-induced mortality 19 attenuates murine autoimmune arthritis by regulation of th17 and treg cells. Arthritis Rheumatol 66: 569–578.

Navarro-Millan I, Singh JA, Curtis JR (2012). Systematic review of tocilizumab for rheumatoid arthritis: a new biologic agent targeting the interleukin-6 receptor. Clin Ther 34: 788–802 e783.

Nixon R, Bansback N, Brennan A (2007). The efficacy of inhibiting tumour necrosis factor alpha and interleukin 1 in patients with

rheumatoid arthritis: a meta-analysis and adjusted indirect comparisons. Rheumatology (Oxford) 46: 1140–1147.

Ohga K, Takezawa R, Arakida Y, Shimizu Y, Ishikawa J (2008a). Characterization of YM-58483/BTP2, a novel store-operated Ca2+ entry blocker, on T cell-mediated immune responses *in vivo*. Int Immunopharmacol 8: 1787–1792.

Ohga K, Takezawa R, Yoshino T, Yamada T, Shimizu Y, Ishikawa J (2008b). The suppressive effects of YM-58483/BTP-2, a store-operated Ca2+ entry blocker, on inflammatory mediator release *in vitro* and airway responses *in vivo*. Pulm Pharmacol Ther 21: 360–369.

Ong HL, Cheng KT, Liu X, Bandyopadhyay BC, Paria BC, Soboloff J *et al*. (2007). Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. J Biol Chem 282: 9105–9116.

Parekh AB (2010). Store-operated CRAC channels: function in health and disease. Nat Rev Drug Discov 9: 399–410.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al*.; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–D1106.

Phillips K, Clauw DJ (2013). Central pain mechanisms in the rheumatic diseases: future directions. Arthritis Rheum 65: 291–302.

Scott DL, Wolfe F, Huizinga TW (2010). Rheumatoid arthritis. Lancet 376: 1094–1108.

Segond von Banchet G, Boettger MK, Fischer N, Gajda M, Brauer R, Schaible HG (2009). Experimental arthritis causes tumor necrosis factor-alpha-dependent infiltration of macrophages into rat dorsal root ganglia which correlates with pain-related behavior. Pain 145: 151–159.

Sheng M, Kim MJ (2002). Postsynaptic signaling and plasticity mechanisms. Science 298: 776–780.

Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD *et al*. (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.

Takezawa R, Cheng H, Beck A, Ishikawa J, Launay P, Kubota H *et al*. (2006). A pyrazole derivative potently inhibits lymphocyte Ca2+ influx and cytokine production by facilitating transient receptor potential melastatin 4 channel activity. Mol Pharmacol 69: 1413–1420.

Vig M, Kinet JP (2009). Calcium signaling in immune cells. Nat Immunol 10: 21–27.

Voelkers M, Salz M, Herzog N, Frank D, Dolatabadi N, Frey N *et al*. (2010). Orai1 and Stim1 regulate normal and hypertrophic growth in cardiomyocytes. J Mol Cell Cardiol 48: 1329–1334.

Wada Y, Nakajima-Yamada T, Yamada K, Tsuchida J, Yasumoto T, Shimozato T *et al*. (2005). R-130823, a novel inhibitor of p38 MAPK, ameliorates hyperalgesia and swelling in arthritis models. Eur J Pharmacol 506: 285–295.

Walsh DA, McWilliams DF (2012). Pain in rheumatoid arthritis. Curr Pain Headache Rep 16: 509–517.

Weber KS, Hildner K, Murphy KM, Allen PM (2010). Trpm4 differentially regulates Th1 and Th2 function by altering calcium signaling and NFAT localization. J Immunol 185: 2836–2846.

Wolfe F, Michaud K, Li T, Katz RS (2010). Chronic conditions and health problems in rheumatic diseases: comparisons with rheumatoid arthritis, noninflammatory rheumatic disorders, systemic lupus erythematosus, and fibromyalgia. J Rheumatol 37: 305–315.

Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S (1997). Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol 121: 417–424.

Xia J, Pan R, Gao X, Meucci O, Hu H (2014). Native store-operated calcium channels are functionally expressed in mouse spinal cord dorsal horn neurons and regulate resting calcium homeostasis. J Physiol 592 (Pt 16): 3443–3461.

Yoshino T, Ishikawa J, Ohga K, Morokata T, Takezawa R, Morio H *et al*. (2007). YM-58483, a selective CRAC channel inhibitor, prevents antigen-induced airway eosinophilia and late phase asthmatic responses via Th2 cytokine inhibition in animal models. Eur J Pharmacol 560: 225–233.

Zatarain E, Strand V (2006). Monitoring disease activity of rheumatoid arthritis in clinical practice: contributions from clinical trials. Nat Clin Pract Rheumatol 2: 611–618.

Zitt C, Strauss B, Schwarz EC, Spaeth N, Rast G, Hatzelmann A *et al*. (2004). Potent inhibition of Ca2+ release-activated Ca2+ channels and T-lymphocyte activation by the pyrazole derivative BTP2. J Biol Chem 279: 12427–12437.