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RESEARCH PAPER

# Cooperative role of tumour necrosis factor-α, interleukin-1β and neutrophils in a novel behavioural model that concomitantly demonstrates articular inflammation and hypernociception in mice

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## BACKGROUND AND PURPOSE

Chronic joint inflammation and pain are the hallmarks of disease in patients with inflammatory arthritis, notably rheumatoid arthritis. The aim of the present study was to investigate the relative contribution of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and neutrophil influx for joint inflammation and nociception in a novel murine model of antigen-induced arthritis (AIA).

## EXPERIMENTAL APPROACH

AIA was induced by administration of antigen into knee joint of previously immunized mice. Neutrophil accumulation was determined by counting neutrophils in the joints and assessing myeloperoxidase activity in tissues surrounding the joints. TNF-α, IL-1β and CXCL-1 were measured by ELISA. Mechanical hypernociception was assessed in parallel, using an electronic pressure meter.

## KEY RESULTS

Hypernociception was dependent on antigen dose and the time after its administration; it was prevented by treatment with morphine and associated with neutrophil infiltration and local production of TNF-α, IL-1β and CXCL-1. Administration of a chimeric monoclonal antibody to TNF-α (infliximab) or IL-1receptor antagonist prevented neutrophil influx and hypernociception, and this was comparable to the effects of dexamethasone. Treatment with fucoidin (a leucocyte adhesion inhibitor) greatly suppressed neutrophil influx and local production of TNF-α and IL-1β, and hypernociception.

## CONCLUSIONS AND IMPLICATIONS

In conclusion, the present study describes a new model that allows for the concomitant evaluation of articular hypernociception and inflammation. Using this system, we demonstrated that a positive feedback loop involving neutrophil influx and the pro-inflammatory cytokines TNF-α and IL-1β is necessary for articular hypernociception after antigen challenge of immunized mice.


### Abbreviations

AIA, antigen-induced arthritis; CFA, complete Freund's adjuvant; IL-1, interleukin-1; IL-1ra, IL-1receptor antagonist; mBSA, methylated BSA; MPO, myeloperoxidase; RA, rheumatoid arthritis; TMB, 3,3′-5,5′-tetramethylbenzidine; TNF-α, tumour necrosis factor-α; TNFR, TNF receptor

## Introduction

Chronic joint inflammation and pain are the two main symptoms in patients with rheumatoid arthritis (RA), and are associated with significant morbidity (Firestein, 2005). RA is a chronic autoimmune disease with repeated acute episodes characterized by infiltration of leucocytes into the synovial and peri-articular tissues (Wipke and Allen, 2001; Liew and McInnes, 2005; Coelho *et al.*, 2008) that results in destruction of cartilage and bone (Firestein, 2003). Neutrophils are the most abundant of leucocytes in the joints of patients with active RA (Kitsis and Weissmann, 1991; Wipke and Allen, 2001), and these cells are thought to contribute to local production of cytokines and to inflict joint damage, perpetuating the inflammatory process (Coelho *et al.*, 2008; Grespan *et al.*, 2008; Lemos *et al.*, 2009). In addition to their potential to inflict joint damage, neutrophils may also play a role in the induction of cutaneous inflammatory pain in animals (Lavich *et al.*, 2006; Cunha *et al.*, 2008).

The sensitization of primary afferent nociceptors is a common denominator of all kinds of inflammatory pain that leads to a state of hyperalgesia and/or allodynia, better described as hypernociception in animal models (Millan, 1999). Hypernociception is induced by inflammatory mediators released in the inflamed tissue in response to a range of inflammatory stimuli that trigger the release of a cascade of cytokines [tumour necrosis factor (TNF)-α, interleukin (IL)-1β and CXC chemokines) by resident and incoming cells (Cunha *et al.*, 2005; Verri *et al.*, 2006). Moreover, cytokines such as TNF-α and IL-1β are also directly implicated in many of the immune processes that are associated with the pathogenesis of RA. Indeed, treatment with anti-TNF antibodies or soluble TNFR has proven an important development in the treatment of patients with RA (Scott and Kingsley, 2006). IL-1β-based therapies also appear to be useful in the context of RA, a tenet that awaits confirmation with drugs or antibodies that are longer acting than anakinra [IL-1receptor antagonist (IL-1ra)]. A problem associated with cytokine-based treatment is that these are costly and need to be given via the parenteral route. Moreover, many patients fail to respond to either TNF-α (Moreland *et al.*, 1997) or IL-1β (Jiang *et al.*, 2000) blockade. Thus, new therapeutic options for the treatment of RA are clearly needed, as are models to investigate joint inflammation and pain concomitantly.

Several experimental models have been used to investigate the pathophysiology of arthritis. However, there are few methods to directly evaluate nociception concomitantly with inflammation events in experimental models of arthritis. In mice, joint hypernociception is usually evaluated only indirectly by stimulating the surrounding tissues in the hind paw after adjuvant administration (Chillingworth and Donaldson, 2003). Pain inferred from performing these latter strategies may reflect sensitization of cutaneous nociceptors instead of joint nociceptive terminals. In the present paper, we addressed the contribution of TNF-α and IL-1β for neutrophil influx and nociception, using a novel behavioural model, and the contribution of neutrophils for cytokine production and nociception.

## Methods

### *Animals*

Animal care and handling procedures were in accordance with the guidelines of the International Association for Study of Pain, and had prior approval from the local animal ethics committee (CETEA, Certificate number 166/2006 and 007/2007). Eight- to ten-week-old male C57Bl/6J (WT) mice were obtained from Centro de Bioterismo of the Universidade Federal de Minas Gerais (UFMG, Brazil) and maintained in the animal facilities of the Laboratório de Imunofarmacologia, Department of Biochemistry and Immunology (UFMG), with filtered water and food *ad libitum* and in a controlled environment (temperature and humidity).

### *Arthritis induction and determination of intra-articular inflammation*

The animals were immunized i.d. at the base of the tail with 500 µg of methylated BSA (mBSA) in 100 µL of an emulsion of saline and an equal volume of complete Freund's adjuvant (CFA) at day 0 (Teixeira *et al.*, 2001). Challenge of mice was performed 14 days later. Each mouse received an injection of the amount of mBSA (in 10 µL sterile saline) in the knee joint. At the indicated time after antigen challenge, the mice were culled. The knee cavity was washed with phosphate-buffered saline (PBS) (2 × 5 µL), and peri-articular tissues were removed for evaluation of cytokines, chemokines and myeloperoxidase (MPO) activity. The total number of leucocytes was determined by counting leucocytes in a Neubauer chamber after staining with Turk's solution. Differential counts were obtained from cytospin (Shandon III, Thermo Shandon, Frankfurt, Germany) preparations by evaluating the percentage of each leucocyte on a slide stained with May–Grünwald–Giemsa.

### *Hypernociception assessment by a modified electronic pressure meter test for mice*

In a quiet room, the mice were placed in acrylic cages (12 × 10 × 17 cm high) with a wire grid floor 15–30 min before testing for environmental adaptation. A series of stimuli was performed only when the animals were quiet, without exploratory movements or defecation and not resting on their paws. In these experiments, an electronic pressure meter was used. It consists of a hand-held force transducer fitted with a polypropylene tip (INSIGHT Instruments, Ribeirão Preto, São Paulo, Brazil) (Cunha *et al.*, 2004). A non-standard large tip (4.15 $mm^2$) and standard large tip (0.5 $mm^2$) were adapted to the probe (Guerrero *et al.*, 2006). An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce the flexion of the knee joint, followed by paw withdraw. A tilted mirror below the grid provided a clear view of the animal's hind paw. The end point was characterized by the removal of the paw. After the flexion-elicited withdrawal threshold, the intensity of the pressure was automatically recorded. The value for the response was obtained by averaging three measurements. The animals were tested before and after treatments. Results are expressed as Δ withdrawal threshold (g) calculated by subtracting zero-time mean measurements from the time interval mean measurements.

The sensitization of the nociceptive neurones in humans results in hyperalgesia (Ferreira, 1972) (an increased response to a stimulus which is normally painful) or allodynia (pain from stimuli that are not normally painful). However, in animal behaviour models of mechanical nociception, hyperalgesia and allodynia can be distinguished by the use of apparently different mechanical tests. Moreover, the terms hyperalgesia and allodynia have been developed for use in clinical practice rather than for experimental work, physiology or anatomical purposes (see IASP Pain Terminology). Therefore, we have used the term hypernociception to describe the decrease of behavioural nociceptive threshold in experimental animals (Parada *et al.*, 2003; Sachs *et al.*, 2004; Cunha *et al.*, 2005; Verri *et al.*, 2006).

### *Quantification of neutrophil accumulation in tissues*

The extent of neutrophil accumulation in tissues was measured by assaying MPO activity (Souza *et al.*, 2002a; Barsante *et al.*, 2007). Briefly, the knee joint was removed and frozen at –70°C. Upon thawing, the tissue (0.1 g of tissue per 1.9 mL of buffer) was homogenized and processed for determination of MPO activity. The assay employed 25 µL of 3,3′-5,5′-tetramethylbenzidine (TMB, Sigma, St Louis, MO, USA) in PBS (pH 5.4) as the colour reagent. Neutrophil number in each sample was calculated from a standard curve of neutrophils obtained from the peritoneal cavity of 5% casein-treated animals, and processed the same way. The results are expressed in relative number of neutrophils $mg^{-1}$ wet tissue. Using this methodology, the test is specific for neutrophils over macrophages and lymphocytes.

### *Measurement of cytokines and chemokines in peri-articular tissues*

The concentrations of TNF-α, IL-1β and CXCL-1 were measured in peri-articular tissues using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet kits, R&D Systems, Minneapolis, MN, USA). Briefly, 100 mg of tissue was homogenized in 1 mL of PBS (0.4 M NaCl and 10 mM de $NaPO_4$) containing antiproteases 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, 20 KI $mL^{-1}$ aprotinin A and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000× *g*, and the supernatant was immediately used for ELISA assay at a 1:3 dilution in PBS. All samples were assayed in duplicate.

### *Histology*

The knee joint was removed and fixed for 24 h with 10% neutral-buffered formalin. The joints were then incubated in 10% EDTA at pH 7.2 for 3 weeks at room temperature to decalcification. The samples were embedded by routine histological technique in

paraffin, and sectioned at 7 μm for Mallory's tricromic staining.

*Drugs.* mBSA, fucoidin, naloxone hydrochloride, morphine sulphate, dexamethasone and CFA were purchased from Sigma Chemical Co., and lidocaine chloride was obtained from Cristália (São Paulo, Brazil). Anti-TNF-α (Infliximab, Remicade®, Kenilworth, NJ, USA) was obtained from Schering-Plough, and IL-1ra was a kind gift from Dr Steve Poole (National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK). All compounds were dissolved in saline. Morphine and naloxone were administered i.p. in a volume of 200 μL. The solution of 2% lidocaine was locally administered in the s.c. plantar tissue in a volume of 10 μL. The abbreviations used conform to *BJP*'s *Guide to Receptors and Channels* (Alexander *et al.*, 2009), and to the IUPHAR guidelines, as published in *Pharmacological Reviews*.

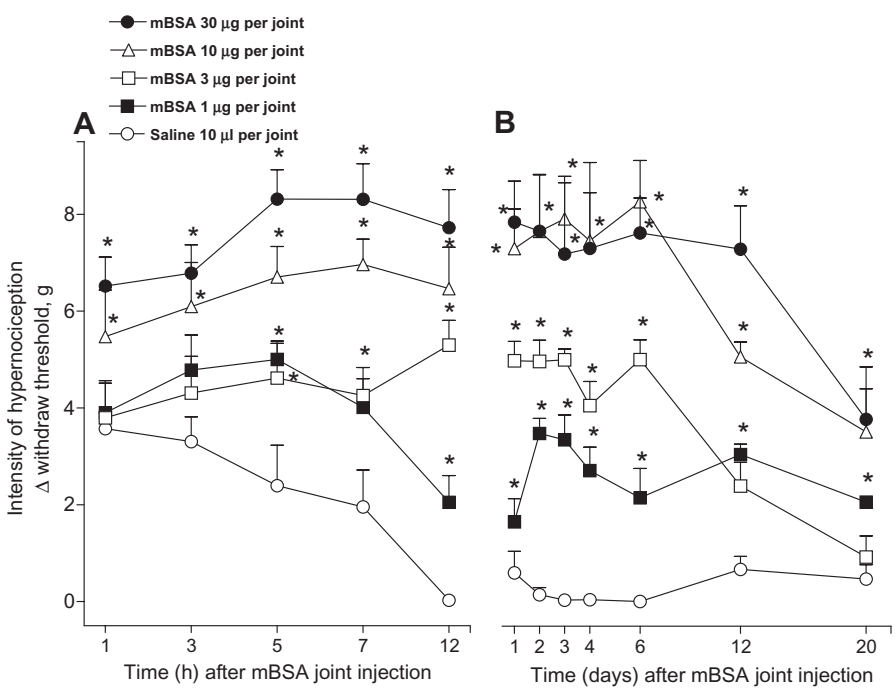
### *Statistical analysis*

Results are shown as the mean ± SEM. Difference among groups was evaluated by using ANOVA followed by Student–Newman–Keuls *post hoc* test. The level of significance was set at $P < 0.05$.

## Results

### *Kinetics of hypernociception in a model of antigen-induced arthritis (AIA)*

The i.a. challenge of immunized mice with 10 μL of sterile saline induced a small degree of hypernociception that peaked at 1 h and declined rapidly thereafter (Figure 1A). Challenge of immunized mice with mBSA induced dose-dependent (1, 3, 10 and 30 μg) mechanical hypernociception that was of much greater intensity and duration. The intensity of hypernociception induced by the lower doses of mBSA (1 and 3 μg i.a.) in immunized mice was significantly increased after 5 h (Figure 1A) of i.a. challenge, remained stable until day 6 and declined thereafter (Figure 1B). For higher doses (10 and 30 μg per cavity), the intensity of hypernociception was significant from the first hour, achieved a maximal response at 5 h and remained at the same magnitude until day 6, declining thereafter (Figure 1), but after each of the assessment times the response was increased when compared with the saline group. A submaximal dose (10 μg) and the read-out at 24 h after i.a. injection of mBSA were chosen for subsequent experiments.




## Figure 1

Dose- and time-dependent mechanical hypernociception after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. mBSA (1, 3, 10 and 30 μg in 10 μL) or sterile saline (control; 10 μL) was injected into the left tibio-femural joint. The intensity of hypernociception was evaluated, 1–12 h (A) and 1–20 days (B) after the injection of mBSA into the joint in immunized mice, by electronic pressure meter test. The results are expressed as the mean ± SEM of 4–5 animals per group. *$P < 0.05$ when compared with control mice.

### *Discriminating articular from cutaneous hypernociception*

A set of experiments was performed to verify whether the probe tip applied on the plantar surface of the hind paw stimulated cutaneous nociceptors instead of articular nociceptors. To distinguish articular (flexion) from cutaneous nociception (poking), two probes with different tip size (standard size tip, 0.5 $mm^2$ and non-standard size tip, 4.15 $mm^2$) were adapted on the electronic pressure meter, and applied on the plantar hind paw surface of the mice. To confirm that the nociceptive response induced by intra-articular administration of mBSA in immunized mice was not due to the sensitization of cutaneous nociceptors, the mice received an intraplantar injection of either lidocaine (2% in 10 µL) or saline (10 µL). As shown in Figure 2, the intraplantar injection of lidocaine prevented the decrease in withdrawal threshold produced by the standard size tip (0.5 $mm^2$, thin tip) and did not alter the flexion movement produced by the large size tip (4.15 $mm^2$, large tip) applied on the plantar surface. These results suggest that the application of the standard size tip provokes cutaneous nociception by itself and that the large tip causes dorsal flexion movement of the knee joint without the stimulation of cutaneous nociceptors, hence allowing the evaluation of the dorsal flexion-elicited hypernociception during joint inflammation. The large size tip (4.15 $mm^2$, large tip) was thus chosen for subsequent experiments.

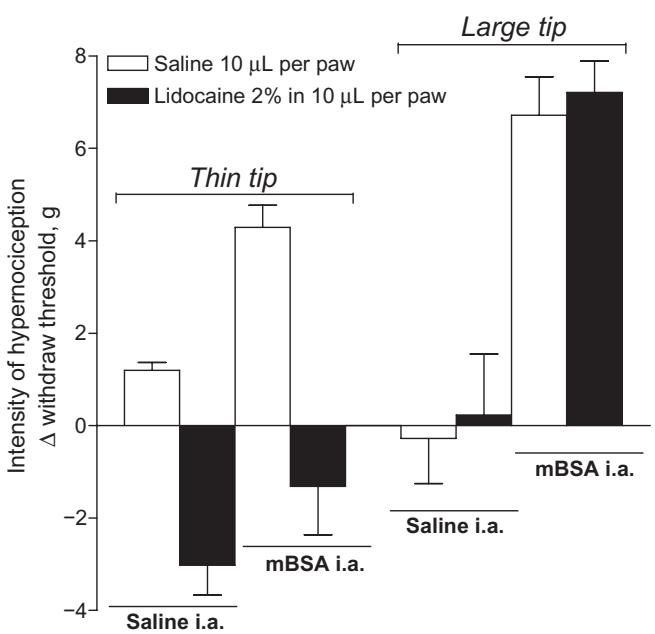



**Figure 2**

Effect of intraplantar lidocaine injection on hypernociceptive responses to injection of antigen (mBSA) in the tibio-femural joint of immunized mice. The mice received an intraplantar injection of lidocaine (Lido: 2%, 10 µL) or saline (10 µL) 24 h after the i.a. injection of mBSA (mBSA, 10 µg in 10 µL) or saline (10 µL). Hypernociception was evaluated 30 min after the intraplantar injection of lidocaine or saline with an electronic pressure meter test using tip probes of two sizes (4.15 and 0.5 $mm^2$). The results are expressed as the mean ± SEM of five animals per group.

### *Effect of morphine on mBSA-induced hypernociception in immunized mice*

To corroborate the view that flexion of tibio-femural joint indicates an articular nociceptive response (not a cutaneous nociceptive response), immunized mice challenged with i.a. mBSA were treated with morphine. The mice received morphine (2–8 $mg \cdot kg^{-1}$; i.p.) 24 h after i.a. injection of mBSA. Treatment with morphine inhibited in a dose-dependent manner the mBSA-elicited hypernociception. Furthermore, treatment with the opioid receptor antagonist naloxone (1 $mg \cdot kg^{-1}$; s.c.) 30 min before morphine (2 $mg \cdot kg^{-1}$; i.p.) injection prevented the analgesic effect of morphine (Figure 3). The administration of naloxone alone had no significant effect on the nociceptive response.

### *Evaluation of joint inflammation*

Joint inflammatory parameters after i.a. challenge of immunized mice with mBSA were also evaluated. There was an increase in the number of neutrophils in the synovial cavity (Figure 4A) and in peri-articular tissues (Figure 4B) at 24 h after challenge. In line with these findings, histological analysis of joint sections demonstrated a dense infiltration of neutrophils in the synovium and peri-articular tissues, and synovial hyperplasia at 24 h after challenge when compared with control (Figure 4C,D). Levels of the cytokines TNF-α and IL-1β, and the chemokine CXCL-1 were increased 24 h after challenge in peri-articular tissues (Figure 5A, B and C respectively).

### *Reduction of mBSA-induced joint inflammation and hypernociception by treatments with anti-TNF-α, IL1-ra and dexamethasone in a model of AIA*

In order to evaluate the usefulness of the model to investigate the role of drugs currently shown to modify the outcome of arthritis, especially TNF-α- and IL-1β-based therapies, we compared the effects of the treatment with anti-TNF-α, IL-1ra and dexamethasone (standard anti-inflammatory steroid) in our model. In the same animals and joints, we assessed the effects of the treatment on hypernociception, and on intra- and peri-articular neutrophil influx. As seen in Figure 6, treatment with anti-TNF-α antibody (infliximab, 10 $mg \cdot kg^{-1}$; s.c.) or

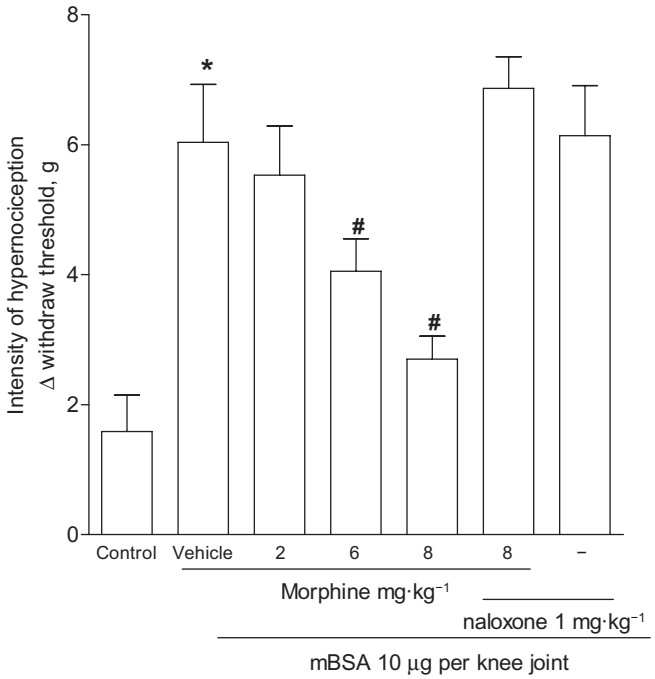



**Figure 3**

Antinociceptive effect of morphine after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. mBSA (10 μg in 10 μL) or sterile saline (control; 10 μL) was injected into the femur-tibial joint. The animals were post-treated (24 h after i.a. injection of mBSA) with morphine (2–8 mg·kg$^{-1}$, i.p.) or vehicle (sterile saline). The effect of naloxone (1 mg·kg$^{-1}$) 30 min before morphine (8 mg·kg$^{-1}$) is also demonstrated. The intensity of hypernociception was evaluated 24 h after i.a. injection of mBSA in immunized mice by electronic pressure meter test. The results are expressed as the mean ± SEM of five animals per group. *$P < 0.05$ when compared with control mice. #$P < 0.05$ when compared to vehicle-treated animals.

IL-1ra (5 mg·kg$^{-1}$; s.c.) 30 min before antigen challenge of immunized mice greatly reduced the hypernociception, and intra- and peri-articular neutrophil influx. Overall, the effect of anti-TNF-α or IL-1ra was of similar magnitude to that of the treatment with dexamethasone (5 mg·kg$^{-1}$; s.c.) (Figure 6). Treatment with anti-TNF-α decreased production of IL-1β by approximately 50%, whereas treatment with IL-1ra decreased TNF-α in the joints by approximately 40% ($n = 4$ in each group, $P < 0.05$).

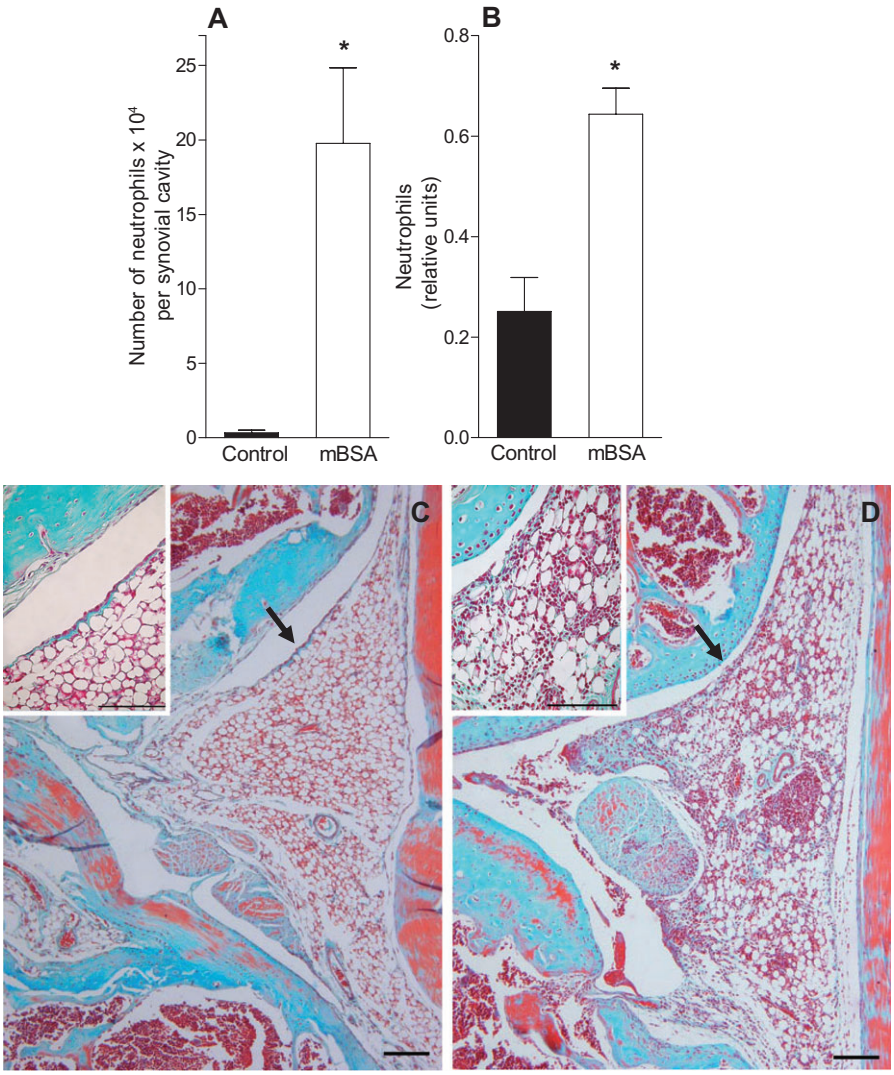
### *Neutrophils play a critical role in mechanical joint hypernociception and cytokine production*

Neutrophils have been shown to play a major role in mechanical and thermal hypernociception due to intraplantar inflammation in rats (Lavich *et al.*, 2006; Cunha *et al.*, 2008). In the present series of experiments, the selectin inhibitor fucoidin 20 (mg·kg$^{-1}$, i.v.) was used to evaluate the role of neutrophil migration in the induction of inflammatory hypernociception. As shown in Figure 7, fucoidin given 10 min before mBSA challenge greatly reduced mechanical hypernociception (Figure 7A), number of neutrophils in the synovial cavity (Figure 7B) and the number of neutrophils in peri-articular tissues (Figure 7C) evaluated 24 h after mBSA challenge in immunized mice. Interestingly, the treatment with fucoidin also reduced concentrations of TNF-α (Figure 7D), IL-1β (Figure 7E), but not CXCL-1 (Figure 7F) in peri-articular tissues.

## Discussion

In the present study, we characterized a novel behavioural model that allows the quantification of articular hypernociception after antigen challenge of immunized mice. Arthritis, induced by administration of antigen into the knee joint of previously immunized mice, mediated a dose-dependent hypernociception, which was associated with a significant infiltrate of inflammatory cells and synovial hyperplasia. Using this model, we show a cooperative role between neutrophil influx and the production of the pro-inflammatory cytokines TNF-α and IL-1β in driving tissue inflammation and hypernociception.

Articular hypernociception is usually indirectly evaluated by mechanical and thermal tests. The application of a cutaneous nociceptive stimulus on the ipsilateral plantar skin is used to detect primary or even secondary hypernociception (central sensitization) induced by inflammatory agents injected in the joint (Butler *et al.*, 1992; Chillingworth and Donaldson, 2003; Cook and Nickerson, 2005). Whereas a standard-sized tip probe evoked a mechanical nociceptive response that was decreased by lidocaine given locally, nociception induced by a large tip probe was not affected by lidocaine application in the paw. These findings further support the notion that in the present model, only articular nociception is being evaluated. Morphine was used to confirm that the decrease of withdrawal threshold elicited by flexion of the knee joint was indicative of a hypernociceptive response, because its analgesic effect has been well documented (Maldonado *et al.*, 1994; Nagakura *et al.*, 2003; Verri *et al.* 2004; 2005). As expected, morphine inhibited in a dose-dependent manner hypernociception after antigen challenge of immunized mice, and this effect was prevented by the opioid receptor antagonist, naloxone. Altogether, these results suggest that the use of a large tip probe in mice is suitable for studying articular hypernociception after antigen challenge of immunized animals.

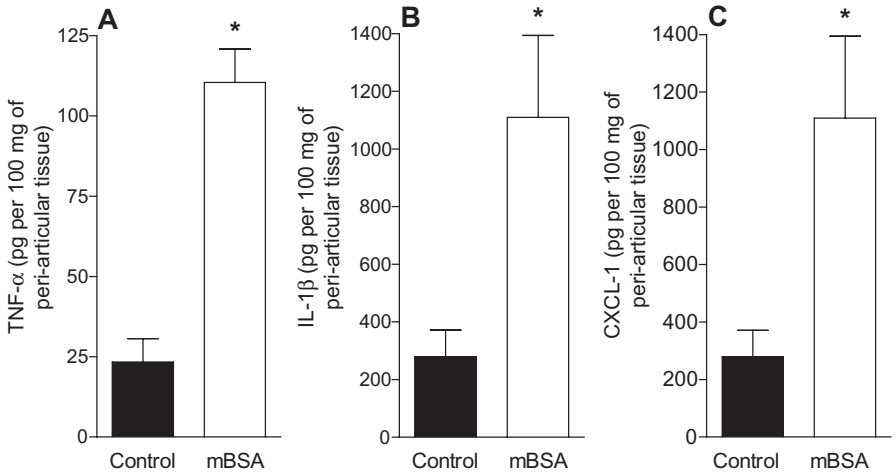



## Figure 4

Articular inflammation after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. mBSA (10 μg in 10 μL) or sterile saline (control; 10 μL) was injected into the right tibio-femural joint. In (A), the number of neutrophils in the synovial cavity was assessed into the knee joint of immunized animals. (B) The number of neutrophils in peri-articular tissues was assessed by using MPO assay, and results are shown as relative units. The results are expressed as the mean ± SEM of five animals per group. *$P < 0.05$ when compared with control mice. Representative sections of knee joints are shown from control (C) or mBSA animals (D) at 24 h after AIA induction or sterile saline injection. Details: high magnification of bone, articular space, synovium and perisinovial tissue (Mallory's trichromic staining); bar = 10 μm. The arrows show the region of greater magnification (×400) that appears as an insert in each of the figures.

Recently, Guerrero *et al.* (2006) evaluated hypernociception evoked by flexion of the tibio-tarsal joint of mice given a non-immune stimulus (zymosan) using a similar methodology. In contrast to our studies, these authors could not evaluate inflammatory parameters (cell influx and cytokine production) concomitantly, due to technical difficulties in obtaining material from the injected joint. Therefore, in addition to using immune stimulation as the cause of joint inflammation, the model described here allows a direct comparison, in the same joint, of leucocyte influx, local cytokine and chemokine production and hypernociception. Therefore, it is possible to evaluate, in the same experimental system, whether the anti-inflammatory effect of a given compound may contribute to its anti-hyperalgesic effect.

Strategies that block or antagonize TNF-α and IL-1β have been used for the treatment of RA, and found to be useful in preventing the progression of the disease in groups of patients (Schiff, 2000; Olsen and Stein, 2004). Consistent with the human studies, treatment of animals with anti-TNF-α, IL-1ra and dexamethasone (standard anti-

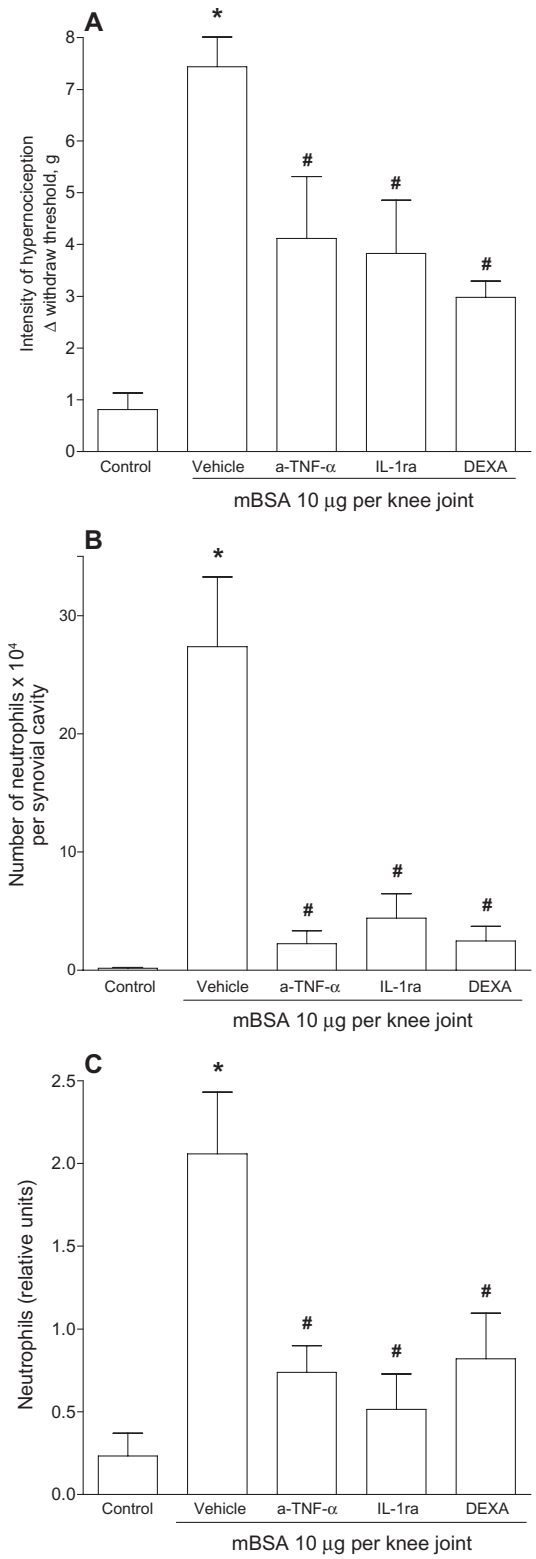



## Figure 5

Levels of TNF-α, IL-1β and CXCL1 in the joint after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. mBSA (10 μg in 10 μL) or sterile saline (control; 10 μL) was injected into the left knee joint. The concentrations of (A) TNF-α, (B) IL-1β and (C) CXCL1 in peri-articular tissues after AIA induction was assessed by ELISA, and results are shown as pg of cytokine/chemokine 100 $mg^{-1}$ tissue. The results are expressed as the mean ± SEM of 4–5 animals per group. *$P < 0.05$ when compared with control mice.

inflammatory steroid) reduced neutrophil migration after antigen challenge of immunized mice. These results demonstrate the relevance of TNF-α and IL-1β for neutrophil influx in this model, and are consistent with the well-known effects of these cytokines in driving the production of a neutrophil chemoattractant and cell adhesion molecule expression at sites of inflammation (Hickey *et al*. 1997; Kelly *et al*. 2007). On the other hand, blockade of neutrophil influx with fucoidin decreased TNF-α and IL-1β production (as seen in Figure 7). Fucoidin inhibits leucocyte migration (Ley *et al*., 1993; Kubes *et al*., 1995; Shimaoka *et al*., 1996; Teixeira and Hellewell, 1997) by binding to L- and P-selectins, and consequently inhibiting leucocyte rolling and subsequent adhesion (Ley *et al*., 1993; Kubes *et al*., 1995; Shimaoka *et al*., 1996). Therefore, in the context of AIA in mice, TNF-α and IL-1β are necessary for an adequate influx of neutrophils, but neutrophils are also essential for the full production of TNF-α and IL-1β. This is not to say neutrophils are actually major producers of these cytokines, but they do appear to contribute to their local production, possibly by interacting with resident macrophages. These findings are consistent with previous findings in another model of acute inflammation induced by reperfusion injury (Souza *et al*., 2000b; 2001; 2002b; 2004). Indeed, in the context of intestinal reperfusion injury, neutrophil influx was necessary for TNF-α production (Souza *et al*., 2000a; 2004), whereas TNF-α was necessary for neutrophil influx (Souza *et al*., 2001). Interestingly, blockade of TNF-α partially decreased IL-1β production, and blockade of IL-1β partially decreased TNF-α production, suggesting that these cytokines facilitate the production of each other in the context of AIA. A previous study from our group showed that CXCR2-acting cytokines, such as CXCL1, are needed for neutrophil influx in AIA (Coelho *et al*., 2008). CXCL1 production was not decreased by fucoidin treatment. Therefore, although CXCL1 acting on CXCR2 appears to be necessary for neutrophil recruitment, it seems that neutrophils are not necessary for the production of CXCL1. Altogether, these studies demonstrate the complexity of the inflammatory response and the many positive feedback loops which are necessary for inflammation to occur. In the context of experimental arthritis, there is strong positive interaction between neutrophils and the pro-inflammatory cytokines TNF-α and IL-1β. Extrapolating this more generally, the existence of positive feedback loops explains why multiple interventions are capable of inhibiting a given inflammatory response, when interventions are given at the beginning of the response.

Several studies have clearly described a role for TNF-α and IL-1β in the genesis of inflammatory hypernociception (Cunha *et al*., 2005; Verri *et al*., 2006). Similarly, it is now clear that neutrophils play a crucial in the induction of inflammatory hypernociception induced by several stimuli (Levine *et al*., 1984; Cunha *et al*., 2008). In our study, strategies that blocked neutrophil influx (fucoidin) or cytokine inhibitors (anti-TNF-α or IL-1ra) decreased hypernociception induced by antigen challenge of immunized mice. These results demonstrate that a positive interaction between neutrophils and cytokines (TNF-α and IL-1β) drives articular inflammation




## Figure 6

Effects of the treatment with anti-TNF-α, IL-1ra or dexamethasone on mechanical hypernociception, neutrophil recruitment and levels of cytokines after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. Anti-TNF-α (10 mg·kg$^{-1}$, s.c.), IL-1ra (5 mg·kg$^{-1}$, s.c.), dexamethasone (DEXA, 5 mg·kg$^{-1}$, s.c.) or vehicle (sterile saline, 200 μL) was given 30 min before mBSA (10 μg, i.a.). Control animals received 10 μL sterile PBS (vehicle) in the knee joint. In (A), the hypernociception was evaluated 24 h after mBSA (10 μg in 10 μL) i.a. injection in immunized mice by electronic pressure meter test. (B) The number of neutrophils in the synovial cavity was assessed in the knee joint of immunized animals. (C) The number of neutrophils in peri-articular tissues was assessed by using MPO assay, and results are shown as relative units. The results are expressed as the mean ± SEM of five animals per group. *$P < 0.05$ when compared with control mice. #$P < 0.05$ when compared to vehicle-treated animals.

after antigen challenge of immunized animals, and triggers the mechanisms that lead to inflammatory hypernociception. However, it is of note that, whereas hypernociception was blocked by around 50% by cytokine inhibitors (or dexamethasone), leucocyte recruitment was inhibited by around 80–90% in the same joint. We have no explanation for these findings, but it is possible that the remaining cell influx may be sufficient for causing a significant degree of hypernociception. In agreement with the latter possibility, fucoidin was the most effective at preventing neutrophil influx and at preventing hypernociception.

In conclusion, we describe a new model that allows for the concomitant evaluation of articular hypernociception and inflammation after antigen challenge of immunized mice. In this model, production of TNF-α, IL-1β and recruitment of neutrophils occur together with the development of articular hypernociception. More importantly, a positive feedback loop involving neutrophil influx and the pro-inflammatory cytokines TNF-α and IL-1β are necessary for the development of articular hypernociception. This model should be useful in the development of novel strategies for the treatment of diseases with joint inflammation and pain, and can be used to evaluate the inhibitory effects of drugs in clinical use on hypernociception.

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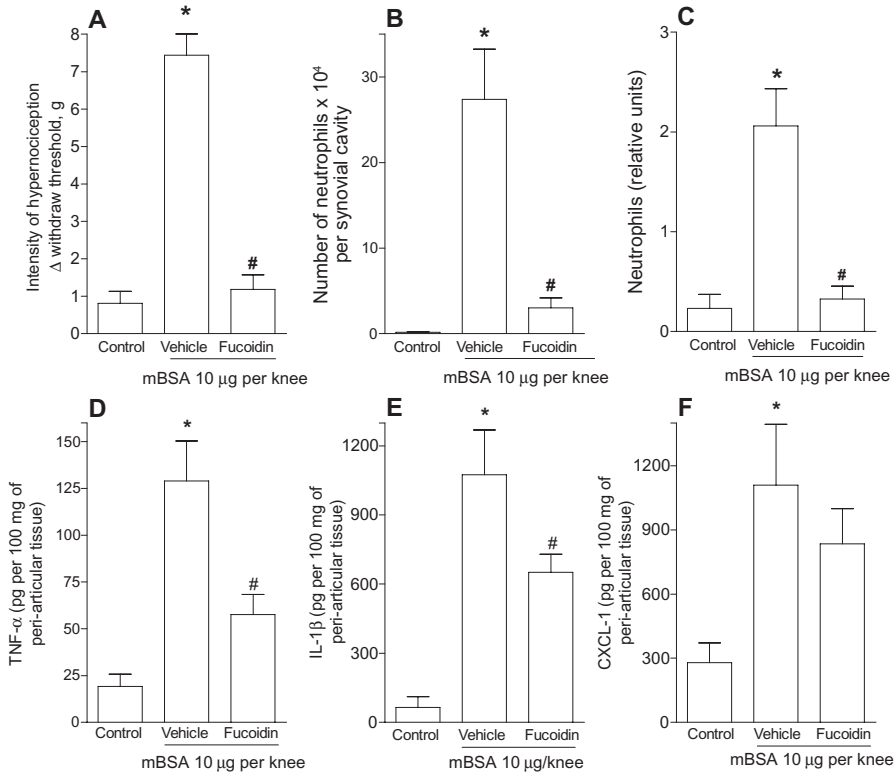
## Conflict of interest

The authors state no conflict of interest.




## Figure 7

Effects of the treatment with fucoidin on mechanical hypernociception, neutrophil recruitment and cytokine production after injection of antigen (mBSA) in the tibio-femural joint of immunized mice. Fucoidin (20 mg·kg$^{-1}$, i.v.) or vehicle (sterile saline, 200 µL) was given 10 min before mBSA (10 µg, i.a.). Control animals received 10 µL sterile PBS (vehicle) in the knee joint. In (A), the hypernociception was evaluated 24 h after mBSA (10 µg in 10 µL) i.a. injection in immunized mice by electronic pressure meter test. (B) The number of neutrophils in the synovial cavity was assessed into the knee joint of immunized animals. (C) The number of neutrophils in peri-articular tissues was assessed by using MPO assay, and results are shown as relative units. The concentrations of (D) TNF-α, (E) IL-1β and (F) CXCL1 in peri-articular tissues after AIA induction were assessed by ELISA, and results are shown as pg of cytokine/chemokine 100 mg$^{-1}$ tissue. The results are expressed as the mean ± SEM of five animals per group. *$P < 0.05$ when compared with control mice. #$P < 0.05$ when compared to vehicle-treated animals.

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