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Toll-like receptor 2/MyD88 signaling mediates zymosan-induced joint hypernociception in mice: Participation of TNF-α, IL-1β and CXCL1/KC

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ABSTRACT

Arthritic pain is a serious health problem that affects a large number of patients. Toll-like receptors (TLRs) activation within the joints has been implicated in pathophysiology of arthritis. However, their role in the genesis of arthritic pain needs to be demonstrated. In the present study, it was addressed the participation of TLR2 and TLR4 and their adaptor molecule MyD88 in the genesis of joint hypernociception (a decrease in the nociceptive threshold) during zymosan-induced arthritis. Zymosan injected in the tibio-tarsal joint induced mechanical hypernociception in C57BL/6 wild type mice that was reduced in TLR2 and MyD88 null mice. On the other hand, zymosan-induced hypernociception was similar in C3H/HePas and C3H/HeJ mice (TLR4 mutant mice). Zymosan-induced joint hypernociception was also reduced in TNFR1 null mice and in mice treated with IL-1 receptor antagonist or with an antagonist of CXCR1/2. Moreover, the joint production of TNF-α, IL-1β and CXCL1/KC by zymosan was dependent on TLR2/MyD88 signaling. Investigating the mechanisms by which TNF-α, IL-1β and CXCL1/KC mediate joint hypernociception, joint administration of these cytokines produced mechanical hypernociception, and they act in an interdependent manner. In last instance, their hypernociceptive effects were dependent on the production of hypernociceptive mediators, prostaglandins and sympathetic amines. These results indicate that in zymosan-induced experimental arthritis, TLR2/MyD88 is involved in the cascade of events of joint hypernociception through a mechanism dependent on cytokines and chemokines production. Thus, TLR2/MyD88 signaling might be a target for the development of novel drugs to control pain in arthritis.

1. Introduction

Rheumatoid arthritis is an autoimmune disease of unknown etiology associated with chronic inflammation of joints and joint pain. Movement-induced joint hyperalgesia is a serious burden to patients presenting inflammatory arthropathies. This clinical state (hyperalgesia) is better defined as hypernociception in experimental models because the majority of experimental nociceptive tests do not differentiate hyperalgesia from allodynia. Recently, we developed an experimental model of inflammatory joint hypernociception in mice, in which the dorsal flexion of inflamed tibio-tarsal joint elicits hypernociception. This method allows direct quantification of this inflammatory symptom (Guerrero et al., 2006). However, the mechanisms of initiation and perpetuation of the inflammatory pain in rheumatoid arthritis are not completely understood.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are involved in the recognition of conserved pathogen-associated molecular patterns (Akira, 2006). They are conserved proteins with an extracellular leucine-rich domain and an intracellular Toll/IL-1 like receptor (TIR) domain. To date, 11 members of the TLR family have been-identified in humans and 13 in mice, being TLR4 the first described in mammals (Akira and Takeda, 2004; Beutler, 2004). Besides to recognize pathogens-associated molecules, there is a novel concept in which endogenous substance could also activates TLRs. For instance, TLR4 has been involved in the recognition of hsp60, fibronectin, heme and multiple host protein (Akira, 2006; Andreakos et al., 2005). Among TLRs, TLR2 and TLR4 have been found highly expressed in synovial tissues of rheumatoid arthritis patients (Radstake et al., 2005). Functionally, genetic or pharmacologic blockade of TLR2 or TLR4 results in the amelioration of arthritis in different experimental models (Seibl et al., 2003; Radstake et al., 2005; Joosten et al., 2003).

The intracellular signaling pathways activated by TLR2 and TLR4 involve the selective recruitment of adapter proteins of which MyD88 is better known. These intracellular cascades lead to the

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activation of transcription factors such as NF-κB, which ultimately trigger the production of inflammatory mediators that might contributes to joint inflammation (Akira and Takeda, 2004). Indeed, it is generally accepted that proinflammatory cytokines (TNF-α and IL-1β) and chemokines (CXCL8/IL-8 and CXCL1/KC) play an important role in the pathogenesis of rheumatoid arthritis (Pierer et al., 2004; Radstake et al., 2005; Haas et al., 2005). Although, a great body of evidence point out these cytokines as important to genesis of inflammatory pain (Cunha et al., 1992, 2005; Verri et al., 2008), their role in the generation of arthritic nociception was not fully addressed. Taking in account these evidences, in the present study, we investigated the role of TLR2, TLR4 and their adapter molecule, MyD88 in the cascade of events involved in the genesis of joint hypernociception during zymosan-induced arthritis. We focused mainly in the role of TLRs triggering cytokine-dependent mechanisms.

2. Materials and methods

2.1. Animals

All experiments were carried out on male C57BL/6 wild type (WT) mice, TNFR1 null mice$^{(-/-)}$, TRL2$^{-/-}$, and MyD88$^{-/-}$ (20–25 g). To investigate the involvement of TLR4, C3H/HeJ mice carrying a dominant-negative mutation in the cytoplasmic domain of TLR4 were used and their littermates C3H/HePas. TNFR1$^{-/-}$, C3H/HePas and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Myd88$^{-/-}$ and TLR2$^{-/-}$ were generated by S. Akira (Osaka University, Japan) and they were a gift to R.T. Gazzinelli. TNFR1$^{-/-}$, Myd88$^{-/-}$, and TLR2$^{-/-}$ were backcrossed at least eight generations into the C57BL/6 background. Mice were housed in the animal care facility of the School of Medicine of Ribeirão Preto. Mice were taken to the testing room at least 1 h before experiments and were used once. All experiments were double blinded and conducted in accordance with the guidelines of the National Institute of Health on the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo.

2.2. Induction of tibio-tarsal joint inflammation

Joint inflammation was induced by administration of zymosan (30 μg) (Guerrero et al., 2006), LPS (300 ng/5 μl), TNF-α (1–1000 pg/5 μl), IL-1β (100–5000 pg/5 μl) and CXCL1/KC (1–100 ng/5 μl) diluted in 5 μl of saline and injected into the right tibio-tarsal joint of mice anesthetized with isoflurane (2%). The substances were injected with a 29 G hypodermic needle inserted into the joint. Control animals received an intra-articular injection of 5 μl sterile saline.

2.3. Articular flexion-elicited hypernociception in the inflamed joint: assessment by a modified electronic pressure-meter test for mice

Experiments were performed as previously described by Guerrero et al. (2006) which is a modification of the test described earlier by Cunha et al. (2004). In a quiet room, mice were placed in acrylic cages (12×10×17 cm high) with a wire grid floor 15–30 min before testing for environmental adaptation. Stimulations were performed only when 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 (IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). An increasing perpendicular force applied to the central area of the plantar surface induced the flexion of the tibio-tarsal joint. A tilted mirror below the grid provided a clear view of the animal's hind paw. In the electronic pressure-meter test, the intensity of the stimulus was automatically recorded when the paw was withdrawn. The non-nociceptive tip probe area size used to evaluate the movement-elicited hypernociception was 4.15 mm^2. The flexion-elicited withdrawal threshold is expressed in grams (g).

2.4. Cytokine and chemokine measurements (ELISA)

At indicated times after the inflammation stimuli injection, animals were killed, and the tibio-tarsal joint regions injected with zymosan or saline were separated from the tibio-tarsal bone complex. The tibio-tarsal joint samples were homogenized in 500 μl of the appropriate buffer containing protease inhibitors, and TNF-α, IL-1β, and KC levels were determined by ELISA (Cunha et al., 2007). The results are expressed as picograms of each cytokine or chemokine per joint.

2.5. Drugs and reagents

Murine recombinant TNF-α, IL-1β, and interleukin 1 receptor antagonist (IL-1ra) were provided by the National Institute for Biological Standards and Control (South Mimms, Herfordshire, UK); recombinant murine CXCL1/KC was purchased from PeproTech (Rocky Hill, NJ); indomethacin (non-selective cyclo-oxigenase inhibitor) was obtained from Prodome (Campinas, SP, Brazil); zymosan, Lipopolysaccharide, (LPS) and guanethidine were obtained from Sigma (St Louis, MO, USA); DF 2162 was obtained from laboratories of Dompé pharma (L'Aquila, Italy).

2.6. Statistical analysis

Results are presented as means ± S.E.M. and are representative of two separate experiments of 5 animals per group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hypernociceptive responses were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Bonferroni's *t* test was performed for each time. Alternatively, when the hypernociceptive responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's *t* test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Participation of TLR2/MyD88 signaling, but not of TLR4, in zymosan-induced joint hypernociception in mice

In the first series of experiments the participation of TLR2 and TLR4, and adaptor molecule MyD88 in zymosan-induced joint hypernociception was investigated using deficient mice. Zymosan injected in C57BL/6 WT mice induced a gradual lowering of the mechanical joint flexion, which was inhibited in TLR2$^{-/-}$ and MyD88$^{-/-}$ mice (Fig. 1A). On contrary, joint administration of zymosan produced hypernociception in C3H/HeJ (TLR4 signaling deficient mice) mice similar to observed in their littermates (C3H/HePas mice) (Fig. 1B). These results suggest that TLR2/MyD88 signaling, but not TLR4, is involved in hypernociceptive response induced by joint injection of zymosan. As control, administration of LPS, a TLR4 agonist, into the joint of C3H/HeJ mice did not induce hypernociception compared with their littermates (C3H/HePas mice; data not shown in fig.).

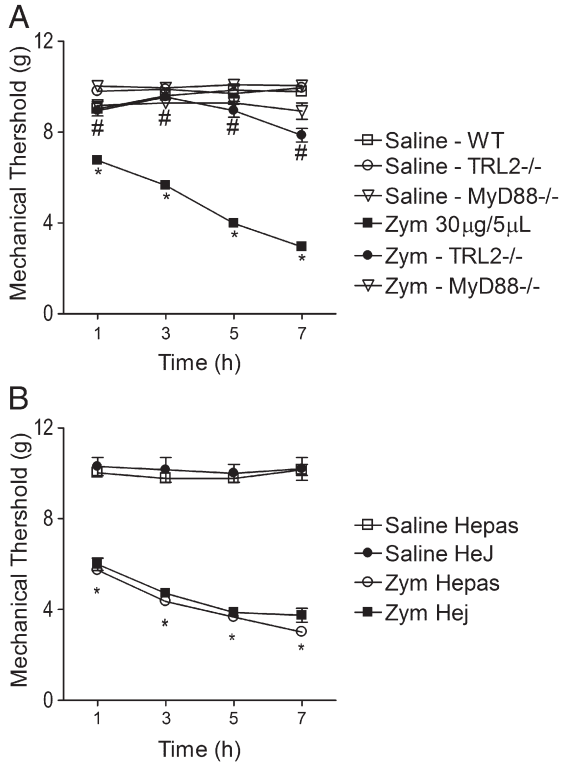

Fig. 1. Role of TLR2, TLR4 and MyD88 in zymosan-induced joint hypernociception. Panel A) zymosan (Zym 30 μg/joint) or saline effect in C57BL/6 wild type (WT), TLR2$^{-/-}$ and MyD88$^{-/-}$. Panel B) Mechanical joint hypernociception was induced by injection of Zym (30 μg/joint) in C3H/HePas and C3H/HeJ mice. Saline was used as a control group. The intensity of hypernociception was evaluated by the mechanical force applied to induce joint flexion measured 1, 3, 5 and 7 h after zymosan tibiotarsal injection. The results are expressed as the mean ± S.E.M. of five animals per group. * means significant difference compared with the saline group. # when compared with zymosan injected group ($P<0.05$).

3.2. Zymosan-induced joint hypernociceptive response was mediated by TNF-α, IL-1β and CXCL1/KC

To further investigate the mechanisms involved in the joint hypernociceptive response induced by zymosan, we evaluated the participation of cytokines TNF-α and IL-β and chemokines (CXCR1/2 ligands, *e.g.* CXCL1/KC) in this response. C57BL/6 WT mice were pretreated with IL-1 receptor antagonist (IL-1ra, 30 mg/kg; i.v, 30 min before zymosan injection, Cunha et al., 2008a; Verri et al., 2008) or with CXCR1/2 antagonist (DF2162, 15 mg/kg; p.o., 60 min before zymosan injection, Cunha et al., 2008b). Both treatments reduced joint hypernociceptive response induced by zymosan (Fig. 2). To evaluate the participation of TNFα, TNFR1$^{-/-}$ mice were used. Joint hypernociception induced by zymosan was also reduced in TNFR1$^{-/-}$ mice compared with C57BL/6 WT mice (Fig. 2). Corroborating these results, joint administration of zymosan in C57BL/6 WT mice induced a time-dependent production of TNFα, IL-1β and CXCL1/KC (Fig. 3A–C). The levels of these cytokines were significant elevated 3 and 5 h after zymosan injection (Fig. 3A–C). These results indicate that TNFα, IL-1β and CXCL1/KC contribute to zymosan-induced joint hypernociceptive response.

3.3. TLR2/MyD88 signaling is involved in cytokines production during zymosan induced joint inflammation

In attempt to evaluate whether cytokines mediation of zymosan induced joint hypernociception is triggered by the activation TLR2/MyD88 signaling, the production of TNFα, IL-1β and CXCL1/KC were evaluated in TLR2$^{-/-}$ and MyD88$^{-/-}$ mice after zymosan joint injection. At 5 h after zymosan joint injection, the production of TNFα, IL-1β and CXCL1/KC were reduced in TLR2$^{-/-}$ mice and they were completely inhibited in MyD88$^{-/-}$ compared with the production in C57BL/6 WT (Fig. 3A–C). These results suggest that TLR2/MyD88 signaling triggers TNF-α, CXCL1/KC and IL-1β in the mediation of zymosan induced joint hypernociception.

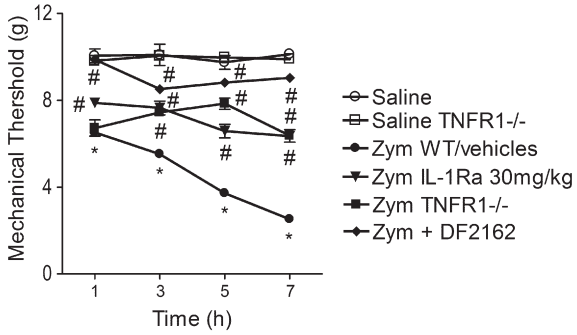

Fig. 2. Participation of TNFα, IL-1β and CXCR1/2 ligands in zymosan-induced joint hypernociception. Mechanical hypernociceptive response induced by joint injection of Zymosan (30 μg/joint) was evaluated in C57BL/6 WT mice pretreated with vehicle, IL-1ra (30 mg/kg, i.v.) and DF2162 (15 mg/kg, p.o) or in TNFR1$^{-/-}$ mice. The hypernociceptive responses were evaluated 1, 3, 5 and 7 h after zymosan intra-articular injection. The results are expressed by the mean ± S.E.M. of five animals per group. The symbol * means significant difference compared with the saline injected group. # when compared with the zymosan injected group treated with vehicle ($P<0.05$).

3.4. Mechanisms involved in cytokine and chemokine mediation of zymosan-induced joint hypernociception

Next, it was investigated the mechanisms involved in cytokine mediation of zymosan-induced joint hypernociception. Firstly, the role of TNF-α was investigated. Intra-articular injection of TNF-α in C57BL/6 WT mice induced mechanical hypernociception in a dose- and time-dependent manner (1–1000 pg/joint) (Fig. 4A). The maximum hypernociceptive response was observed with the dose of 100 pg of TNF-α. The hypernociception was already significant 1 h after TNF-α injection, reached a plateau at 5 h after injection, and returned to control levels 24 h after injection (Fig. 4A). The joint hypernociceptive effect of TNF-α was reduced by the pretreatment of C57BL/6 WT mice with a non-selective inhibitor of cyclooxigenase (indomethacin, 5 mg/kg, i.p. 30 min before TNF-α injection; Verri et al., 2006) with a sympathomimetic neuron-blocking agent (guanethidine, 30 mg/kg, s.c., 30 min before TNF-α injection; Pinto et al., 2010) and it was inhibited by the association of indomethacin and guanetidine (Fig. 4B). In addition, the pretreatment of C57BL/6 WT with IL-1Ra (30 mg/kg, i.v., 30 min before TNF-α injection) and DF2162 (15 mg/kg, p.o., 60 min before TNF-α injection) also reduced TNF-α-induced joint hypernociception (Fig. 4B). Confirming these results, the joint injection of TNF-α increased the production of CXCL-1/KC (Fig. 4C) and IL-1β (Fig. 4D). The participation of TNF-α in the induction of CXCL-1/KC and IL-1β during zymosan-induced joint inflammation was confirmed by the fact that the production of CXCL-1/KC and IL-1β were reduced in TNFR1$^{-/-}$ mice (Fig. 4 C and D).

Next, the role of IL-1 β was evaluated. Intra-articular injection of IL-1β induced joint hypernociception in a dose- and time-dependent manner (100, 1000 and 5000 pg/joint) (Fig. 5A). The maximum hypernociceptive response was observed with the dose of 5000 pg of IL-1β. The hypernociception was already significant 1 h after IL-1β injection, reached a plateau at 5 h after articular injection (Fig. 5A). The joint hypernociceptive effect of IL-1β was reduced by the pretreatment of C57BL/6 WT mice with indomethacin or guanethidine, and it was inhibited by the association of indomethacin and guanethidine (Fig. 5B). In addition, the pretreatment of C57BL/6 WT with DF2162 also reduced IL-1β-induced joint hypernociception

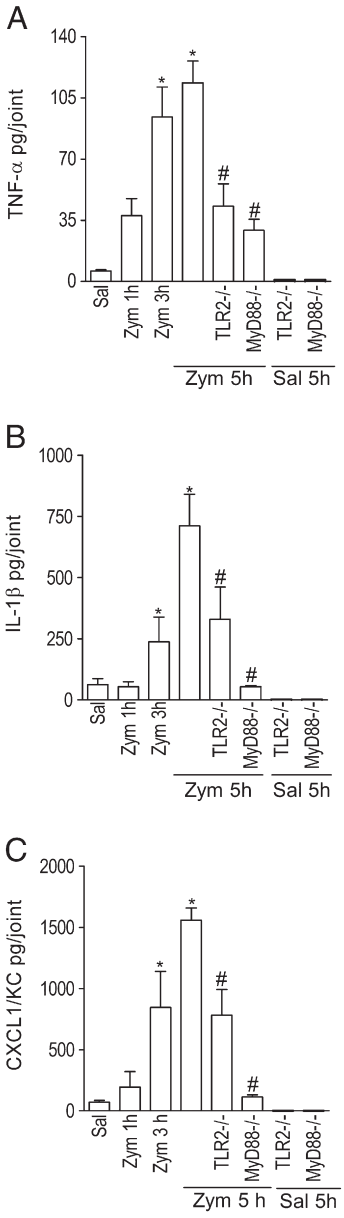

Fig. 3. Articular injection of zymosan increases the production of TNF-α, IL-1β and CXCL1/KC in joint tissue: role of TLR2/MyD88 signaling. Panel A: Concentration of TNF-α in MyD88$^{-/-}$ and TLR2$^{-/-}$ deficient mice and WT mice joint injected with of zymosan or saline (Sal). At 5 h after intra-articular injection, mice were killed and joint tissue samples were extracted for cytokines, which were measured by ELISA. Panel B: Concentration of IL-1β in MyD88$^{-/-}$ and TLR2$^{-/-}$ deficient mice and WT mice joint injected with of zymosan or saline. At 5 h after intra-articular injection, mice were killed and joint tissue samples were extracted for cytokines, which were measured by ELISA. Panel C: Concentration of CXCL1/KC in MyD88$^{-/-}$ and TLR2$^{-/-}$ mice and WT mice joint injected with zymosan or saline. At 5 h after intra-articular injection, mice were killed and joint tissue samples were extracted for cytokines, which were measured by ELISA. The results are expressed by the mean ± S.E.M. of five animals per group. The symbol * means significant difference when compared with the saline group. # when compared with the zymosan injected in WT mice group ($P<0.05$).

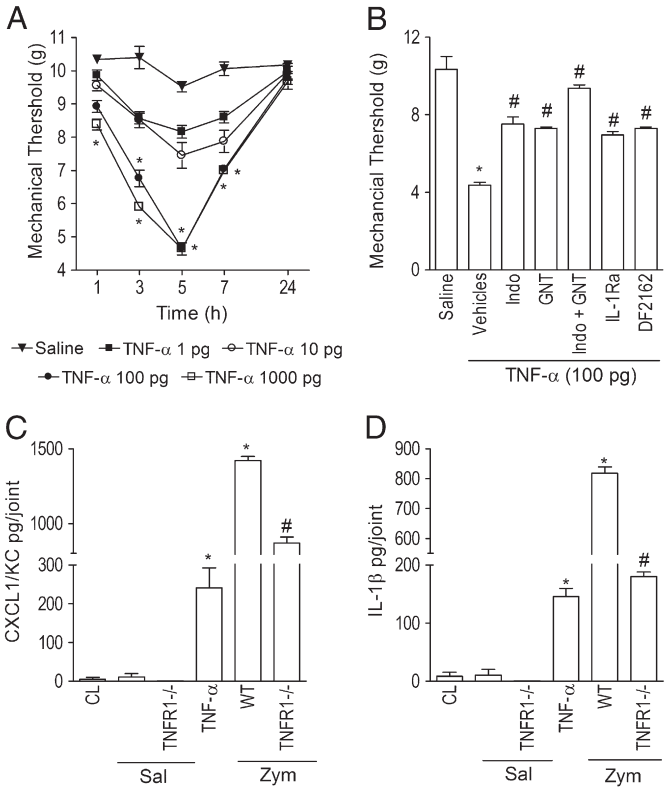

Fig. 4. TNF-α induced tibio-tarsal joint hypernociception. Panel A: Dose and time–response curves of the hypernociception induced by intra-articular injection of TNF-α (1, 10, 100 and 1000 pg/5 μl) or saline. The hypernociceptive effects were determined at 1, 3, 5, 7 and 24 h after intra-articular injection of TNF-α. Panel B: Nociceptive response induced by TNF-α (100 pg/5 μl) WT mice pre-treatment with IL-1ra (30 mg/kg, i.v.), DF2162 (15 mg/kg p.o., indomethacin (indo, 5 mg/kg i.pl.) guanethidine (GNT, 30 mg/kg s.c.) and indomethacin plus guanethidine. Panel C: Joint production of CXCL1/KC in mice after intra-articular injection of TNF-α (100 pg/5 μl) and Zym (30 μg/5 μl) in WT and in TNFR1$^{-/-}$ mice. Panel D: Joint production of IL-1β in mice after intra-articular injection of TNF-α (100 pg/5 μl) and Zym (30 μg/5 μl) in WT and in TNFR1$^{-/-}$ mice. The results are expressed by the mean ± S.E.M. of five animals per group. The symbol * means significant difference when compared with the saline group. # when compared with the zymosan injected in WT mice group ($P<0.05$).

(Fig. 5B). Moreover, the hypernociceptive response induced by IL-1β was also reduced in TNFR1$^{-/-}$ mice (Fig. 5B). Further supporting these results, joint injection of IL-1β increased the production of TNF-α and CXCL1/KC in the joint of mice (Fig. 5C and D, respectively). Moreover, the production of TNF-α and CXCL1/KC induced by zymosan joint injection was reduced by the treatment of mice with IL-1ra (Fig. 5 C and D, respectively).

Finally, the role of CXCL-1/KC in the mediation of zymosan-induced joint hypernociception was investigated. Intra-articular injection of CXCL1/KC induced joint hypernociception in a dose- and time-dependent manner (1–100 ng/joint; Fig. 6A). The maximum hypernociceptive response was observed with the dose of 30 ng of CXCL1/KC. The joint hypernociception was already significant 1 h after CXCL1/KC injection, reached a plateau at 5 h after joint injection (Fig. 6A). The joint hypernociceptive effect of CXCL1/KC was reduced by the pretreatment of C57BL/6 WT mice with indomethacin or guanethidine, and it was inhibited by the association of indomethacin and guanethidine (Fig. 6B). In addition, the pretreatment of C57BL/6 WT with IL-1ra also reduced CXCL1/KC-induced joint hypernociception (Fig. 6B). Moreover, the joint hypernociceptive response induced by CXCL1/KC was reduced in TNFR1$^{-/-}$ mice (Fig. 6B). In agreement, joint injection of CXCL1/KC increased the production of IL-1β and TNF-α (Fig. 6C and D, respectively). Moreover, zymosan induced the production of TNF-α and CXCL1/KC in C57BL/6 WT which was reduced by the treatment with DF2162 (Fig. 6C and D, respectively)

4. Discussion

Arthritic pain is a serious health problem that affects a large number of patients. Therefore, the knowledge of the underlying mechanisms involved in its genesis could lead to the development of novel and effective pharmacological therapies. In the present study, we investigated the role of TLRs and their signaling in the cascade of events that mediate arthritic nociception in zymosan-induced arthritis. We

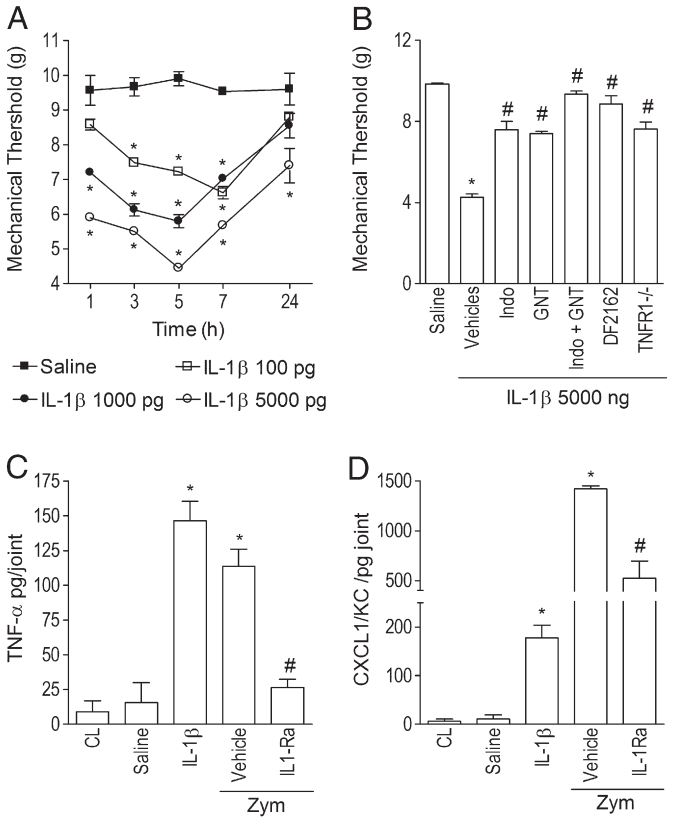

Fig. 5. IL-1β induced tibio-tarsal joint hypernociception. Panel A: Dose and time–response curves of the hypernociception induced by intra-articular injection of IL-1β (100, 1000 and 5000 pg/5 μl) or saline. The hypernociceptive effects were determined at 1, 3, 5, 7 and 24 h after intra-articular injection of IL-1β. Panel B: Nociceptive response induced by IL-1β (5000 pg/5 μl) in TNFR1$^{-/-}$ mice or in WT mice pretreated with DF2162 (15 mg/kg delivered p.o.) indomethacin (indo, 5 mg/kg delivered i.pl.) guanethidine (GNT, 30 mg/kg delivered s.c.) and indomethacin plus guanethidine. Panel C: Joint production of TNF-α in mice after intra-articular injection of IL-1β (5000 pg/5 μl) and Zym (30 μg/5 μl) in vehicle an IL-1ra (30 mg/kg, i.v.) treated mice. Panel D: Joint production of CXCL1/KC in mice after intra-articular injection of IL-1β (5000 pg/5 μl) and Zym (30 μg/5 μl) in vehicle and IL-1ra treated mice. The results are expressed by the mean ± S.E.M. of five animals per group. The symbol * means significant difference when compared with the saline group. # when compared with the IL-1ra treated group ($P<0.05$).

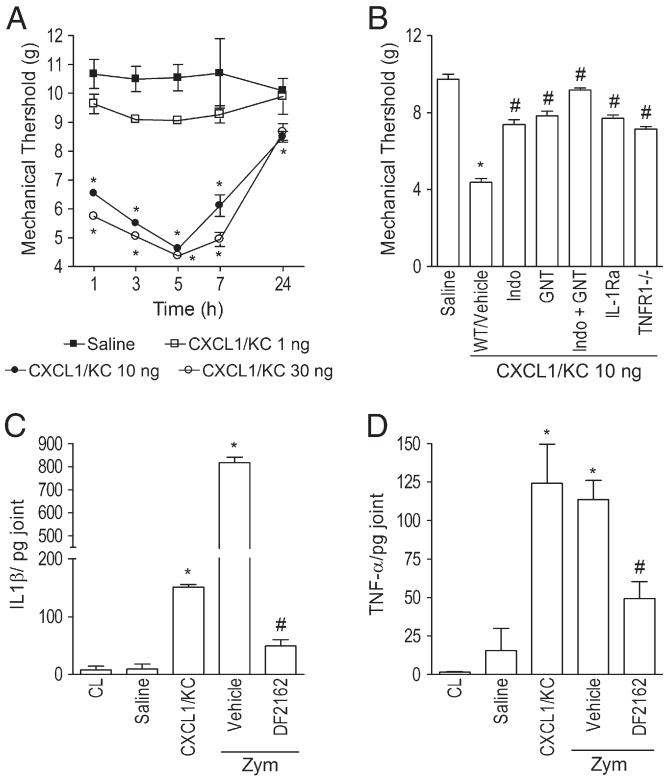

Fig. 6. CXCL1/KC induced tibio-tarsal joint hypernociception. Panel A: Dose and time–response curves of the hypernociception induced by intra-articular injection of CXCL1/KC (1, 10 and 30 ng/5 μl) or saline. The hypernociceptive effects were determined at 1, 3, 5, 7 and 24 h after intra-articular injection of CXCL1/KC. Panel B: Nociceptive response induced by CXCL1/KC (10 ng/5 μl) in TNFR1$^{-/-}$ mice and in WT mice pretreated with IL-1ra (30 mg/kg, i.v.), indomethacin (indo, 5 mg/kg i.pl.) guanethidine (GNT, 30 mg/kg s.c.) and indomethacin plus guanethidine. Panel C: Joint production of TNF-α in mice after intra-articular injection of CXCL1/KC (10 ng/5 μl), and Zym (30 μg/5 μl) in vehicle and DF2162 (15 mg/kg, p.o.) treated mice. Panel D: Joint production of IL-1β in mice after intra-articular injection of CXCL1/KC (10 ng/5 μl), and Zym (30 μg/5 μl) in vehicle and DF2162 (15 mg/kg, p.o.) treated mice. The results are expressed by the mean ± S.E.M. of five animals per group. The symbol * means significant difference when compared with the saline group. # when compared with DF2162 treated group ($P<0.05$).

are showing that zymosan-induced joint hypernociception depends on activation of TLR2/MyD88, but not TLR4 signaling pathway, those result in the production of cytokines (TNF-α, IL-1β and CXCL1/KC) which acts in a reciprocal/self-stimulatory cytokine cascade. Probably these cytokines lead to the production of prostanoids and sympathetic amines that ultimately sensitize the primary nociceptive neurons as observed in other models of inflammation (Cunha et al., 2005; Guerrero et al., 2006).

There are previous evidences that TLR2 and TLR4 are functionally expressed in synovial tissue of patients with rheumatoid arthritis and their activation leads to the production of several inflammatory mediators (De Rycke et al., 2005; Iwahashi et al., 2004; Pierer et al., 2004; Radstake et al., 2005). In this context, TLR2 and TLR4 deficient mice present reduced arthritis in a range of experimental models, either in auto-immune or infections triggered arthritis (Joosten et al., 2003; Liu-Bryan et al., 2005). The current hypothesis is that endogenous produced or pathogen-derived molecules could activate synovial TLRs participating in the physiopathology of those diseases (Roelofs et al., 2005). Here, we extend these studies presenting evidence that TLR2/MyD88 signaling, but not TLR4, is involved in the induction of articular nociception in zymosan-induced arthritis. Our result is in accordance with previous evidence showing that zymosan, at least *in vitro*, directly activates TLR2 but not TLR4 (Lamkanfi et al., 2009). Besides to act as TLR2 agonist, zymosan might also stimulate endogenous agonists of TLR2 receptors such as heat shock proteins which could amplify the inflammatory process (Roelofs et al., 2005). Although TLR4 did not participate in joint hypernociception during zymosan-induced arthritis, it could contribute to pain in other models of arthritis, including in LPS-induced joint inflammation and also in humans. Moreover, it was recently showed that the activation of TLR4 by an endogenous mediator play a critical role in the persistence of synovial inflammation and tissue destruction in the chronic phase of zymosan-induced arthritis (Midwood et al., 2009).

The hypernociceptive mechanisms triggered by the activation of TLR2/MyD88 signaling during zymosan-induced arthritis seems to be dependent on the stimulation of the production of pronociceptive cytokines and chemokines, including TNF-α, IL-1β and CXCL1, respectively. Previous studies have demonstrated that TLR-2/MyD88 signaling lead to the activation of NF-κB (Andreakos et al., 2005) in a mechanism dependent on MAP kinases (Takeda and Akira, 2005). Our findings suggested that joint of TLR2$^{-/-}$ and MyD88$^{-/-}$ mice injected with zymosan exhibited decrease levels in the production of TNF-α, IL-1β and CXCL1/KC. It was interesting that although the production of these cytokines was reduced in TLR2$^{-/-}$, the reductions were more pronounced in MyD88$^{-/-}$, suggesting that a mechanism other than activation of TLR2 might account for the stimulation of MyD88 pathway and the production of cytokines (Joosten et al., 2003). Maybe the activation of other TLRs

or even the activation of IL-1β/IL-1R might potentiate MyD88-dependent events.

The production of TNF-α, IL-1β and CXCL8/IL-8 (counterpart of mouse CXCL1/KC) in synovial tissue of patients with rheumatoid arthritis appears to be an important process in the pathogenesis of this auto-immune disease (Torres et al., 2009). Experimentally, these cytokines and chemokines play a critical role in the manifestation of inflammatory hypernociception in vast number of experimental models (Cunha et al., 2005, 2008a, 2008b; Verri et al., 2006). For instance, genetic or pharmacological inhibition of TNFR1, IL-1R1 and CXCR2, the respective receptor for TNFα, IL-1β and CXCL1/KC reduced articular hypernociception in a model antigen-induced arthritis (Verri et al., 2008). In the present study, we further elucidate the relationship among these cytokines in producing joint hypernociception. Although the group of cytokines that mediates paw inflammation-induced hypernociception is the same detected in zymosan-induced joint hypernociception, the mechanism operating their action seems to be quite different. Indeed, differently from we have demonstrated in the plantar hypernociception of rats and mice in which inflammation induced the production of a sequential cascade of pro-hypernociceptive cytokines (Cunha et al., 1992, 2005), in the joint inflammation, it seems that these cytokines are released in an interdependent manner, but not sequentially. These results indicate that targeting 1 of these cytokines could break the cycle and reduce joint hypernociception. It is obvious that these cytokines/chemokines have other roles in joint tissue pathophysiology of rheumatoid arthritis, and is up to us to determine the best target with reduced side-effect incidence. One important fact is that zymosan-induced hypernociception at earlier time point (1 h after) seems to be not mediated by these cytokines. In fact, mediators such as leukotriene B4 (Guerrero et al., 2008), platelet activating factor (Guerrero et al., unpublished results) and C5a (Ting et al., 2008) might be involved in this zymosan effect.

It is important to point out that the hypernociceptive action of cytokines in the joint, as observed in the paw (Cunha et al., 2005), is indirectly, via the production of directly acting hypernociceptive mediators such as prostaglandins and sympathetic amines. Nonetheless, there are evidences that cytokines could be acting directly on primary nociceptive neurons promoting their sensitization (Richter et al., 2010). The *in vivo* importance of directly or indirectly action of cytokines for the induction of joint hypernociception still needs to be demonstrated, for example, by the use of conditional mice with deletion of cytokines receptors only in primary nociceptive neurons.

Besides the involvement of local (joint) activation of TLR2 in the induction of arthritis pain, there is recent data suggesting that the chronification of pain process, at least during neuropathic process after peripheral nerve injury, depends on TLR2 expressed in the central nerve system (Kim et al., 2007). It seems that there are molecules released by injured neurons that activate TLR2 (Kim et al., 2007). Furthermore, the stimulation of TLR2 in microglial cell promotes their activation and contributes to the maintenance of neuropathic pain (Kim et al., 2007). Therefore, it is plausible to suggest that the chronification of pain in arthritis, in which microglia activation also plays a role (Sun et al., 2007), could be dependent on spinal activation of TLR2 signaling.

Our study presents an insight in the mechanisms that trigger arthritic pain, demonstrating that the activation TLR2/MyD88 signaling plays an important role. The TLR2/MyD88 signaling drives the production of pronociceptive cytokines including TNF-α, IL-1β and CXCL1/KC, which act in an interdependent manner, one releasing each other. In the joint, the hypernociceptive effect of cytokines is also indirect through the stimulation of directly sensitizing mediators (prostanoids and sympathetic amines), which might sensitize primary nociceptive neurons leading to joint hypernociception (Fig. 7). This process is in line with the explanation that in arthritis analgesia can be achieved by the use of corticoids and selective inhibitor of cytokines (human antibodies) that blockade the release/action of hyperalgesic cytokines and non steroid anti-inflammatory drugs (NSAID), which prevent prostanoids-induced nociceptor sensitization. In conclusion, the present results indicate that the development of inhibitors of TLR2/MyD88 or downstream (cytokines/chemokines) signaling could be an alternative to control arthritic pain.

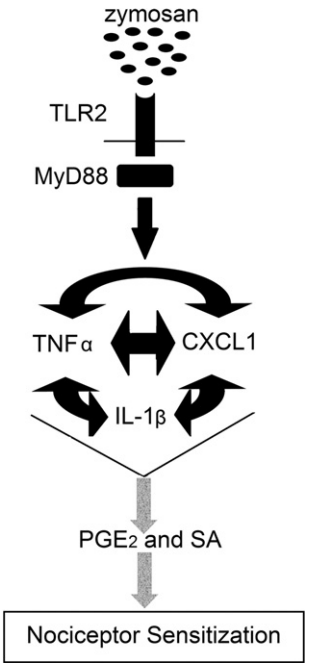

Fig. 7. Representative diagram of the mechanism underling the TLR2 mediation of hypernociception in zymosan-induced arthritis.The fig. summarizes the essential conclusion of the present study, which shows the role of TLR2/MyD88 signaling in arthritic pain by the stimulation of TNF-α, IL-1β and CXCL1/KC, which in turn promote the production of prostaglandins and sympathetic amines (SA).

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References

Akira, S., 2006. TLR signaling. Curr. Top. Microbiol. Immunol. 311, 1–16.
Akira, S., Takeda, K., 2004. Functions of toll-like receptors: lessons from KO mice. C. R. Biol. 327, 581–589.
Andreakos, E., Sacre, S., Foxwell, B.M., Feldmann, M., 2005. The toll-like receptor-nuclear factor kB pathway in rheumathoid arthritis. Front. Biosci. 10, 2478–2488.
Beutler, B., 2004. Toll-like receptors and their place in immunology. Where does the immune response to infection begin? Nat. Rev. Immunol. 4, 498.
Cunha, F.Q., Poole, S., Lorenzetti, B.B., Ferreira, S.H., 1992. The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. Br. J. Pharmacol. 107, 660–664.
Cunha, T.M., Verri Jr., W.A., Vivancos, G.G., Moreira, I.F., Reis, S., Parada, C.A., Cunha, F.Q., Ferreira, S.H., 2004. An electronic pressure-meter nociception paw test for mice. Braz. J. Med. Biol. Res. 37, 401–417.
Cunha, T.M., Verri Jr., W.A., Silva, J.S., Poole, S., Cunha, F.Q., Ferreira, S.H., 2005. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proc. Natl. Acad. Sci. U. S. A. 102, 1755–1760.
Cunha, T.M., Verri Jr., W.A., Fukada, S.Y., Guerrero, A.T., Santodomingo-Garzón, T., Poole, S., Parada, C.A., Ferreira, S.H., Cunha, F.Q., 2007. TNF-alpha and IL-1beta mediate inflammatory hypernociception in mice triggered by B1 but not B2 kinin receptor. Eur. J. Pharmacol. 573, 221–229.

Cunha, T.M., Verri Jr., W.A., Valério, D.A., Guerrero, A.T., Nogueira, L.G., Vieira, S.M., Souza, D.G., Teixeira, M.M., Poole, S., Ferreira, S.H., Cunha, F.Q., 2008a. Role of cytokines in mediating mechanical hypernociception in a model of delayed-type hypersensitivity in mice. Eur. J. Pain 12, 1059–1068.

Cunha, T.M., Barsante, M.M., Guerrero, A.T., Verri Jr., W.A., Ferreira, S.H., Coelho, F.M., Bertini, R., Di Giacinto, C., Allegretti, M., Cunha, F.Q., Teixeira, M.M., 2008b. Treatment with DF 2162, a non-competitive allosteric inhibitor of CXCR1/2, diminishes neutrophil influx and inflammatory hypernociception in mice. Br. J. Pharmacol. 154, 460–470.

De Rycke, L., Vandooren, B., Kruithof, E., De Keyser, F., Veys, E.M., Baeten, D., 2005. Tumor necrosis factor α blockade treatment down-modulates the increase systemic and local expression of toll-like receptors 2 and toll-like receptors 4 in spondylarthropathy. Arthritis Rheum. 52, 2146–2158.

Guerrero, A.T., Verri Jr., W.A., Cunha, T.M., Silva, T.A., Rocha, F.A., Ferreira, S.H., Cunha, F.Q., Parada, C.A., 2006. Hypernociception elicited by tibio-tarsal joint flexion in mice: a novel experimental arthritis model for pharmacological screening. Pharmacol. Biochem. Behav. 84, 244–251.

Guerrero, A.T., Verri Jr., W.A., Cunha, T.M., Silva, T.A., Schivo, I.R., Dal-Secco, D., Canetti, C., Rocha, F.A., Parada, C.A., Cunha, F.Q., Ferreira, S.H., 2008. Involvement of LTB4 in zymosan-induced joint nociception in mice: participation of neutrophils and PGE2. J. Leukoc. Biol. 83, 122–130.

Haas, C.S., Martinez, R.J., Attia, N., Haines III, G.K., Campbell, P.L., Koch, A.E., 2005. Chemokine receptor expression in rat adjuvant-induced arthritis. Arthritis Rheum. 52, 3718–3730.

Iwahashi, M., Yamamura, M., Aita, T., Okamoto, A., Ueno, A., Ogawa, N., Akashi, S., Miyake, K., Godowski, P.J., Makino, H., 2004. Experession of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumathoid arthritis. Arthritis Rheum. 50, 1457–1467.

Joosten, L.A., Koenders, M.I., Smeets, R.L., Heuvelmans-Jacobs, M., Helsen, M.M., Takeda, K., Akira, S., Lubberts, E., van de Loo, F.A., van den Berg, W.B., 2003. Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J. Immunol. 171, 6145–6153.

Kim, D., Kim, M.A., Cho, I.H., Kim, M.S., Lee, S., Jo, E.K., Choi, S.Y., Park, K., Kim, J.S., Akira, S., Na, H.S., Oh, S.B., Lee, S.J., 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J. Biol. Chem. 282, 14975–14983.

Lamkanfi, M., Malireddi, R.K., Kanneganti, T.D., 2009. Fungal zymosan and mannan activate the cryopyrin inflammasome. J. Biol. Chem. 31, 20574–20581.

Liu-Bryan, R., Scott, P., Sydlaske, A., Rose, D.M., Terkeltaub, R., 2005. Innate immunity conferred by toll-like receptors 2 and 4 and myeloid differentiation factor 88 expression is pivotal to monosodium urate monohydrate crystal-induced inflammation. Arthritis Rheum. 52, 2936–2946.

Midwood, K., Sacre, S., Piccinini, A.M., Inglis, J., Trebaul, A., Chan, E., Drexler, S., Sofat, N., Kashiwagi, M., Orend, G., Brennan, F., Foxwell, B., 2009. Tenascin-C is an endogenous activator of toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat. Med. 15, 774–780.

Pierer, M., Rethage, J., Seibl, R., Lauener, R., Brentano, F., Wagner, U., Hantzschel, H., Michel, B.A., Gay, R.E., Gay, S., Kyburz, D., 2004. Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. J. Immunol. 172, 1256–1265.

Pinto, L.G., Cunha, T.M., Vieira, S.M., Lemos, H.P., Verri Jr., W.A., Cunha, F.Q., Ferreira, S.H., 2010. IL-17 mediates articular hypernociception in antigen-induced arthritis in mice. Pain 148, 247–256.

Radstake, T.R., Roelofs, M.F., Jenniskens, Y.M., Oppers-Walgreen, B., van Riel, P.L., Barrera, P., Joosten, L.A., van den Berg, W.B., 2005. Expression of Toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflamatory cytokines interleukin-12 and interleukin-18 via interferon-γ. Arthritis Rheum. 50, 3856–3865.

Richter, F., Natura, G., Löser, S., Schmidt, K., Viisanen, H., Schaible, H.G., 2010. Tumor necrosis factor causes persistent sensitization of joint nociceptors to mechanical stimuli in rats. Arthritis Rheum. 62, 3806–3814.

Roelofs, M.F., Joosten, L.A., Abdollahi-Roodsaz, S., van Lieshout, A.W., Sprong, T., van den Hoogen, F.H., van den Berg, W.B., Radstake, T.R., 2005. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis Rheum. 52, 2313–2322.

Seibl, R., Birchler, T., Loeliger, S., Hossle, J.P., Gay, R.E., Saurenmann, T., Michel, B.A., Seger, R.A., Gay, S., Lauener, R.P., 2003. Expression and regulation of toll-like receptor 2 in rheumatoid arthritis synovium. Am. J. Pathol. 162, 221–227.

Sun, S., Cao, H., Han, M., Li, T.T., Pan, H.L., Zhao, Z.Q., Zhang, Y.Q., 2007. New evidence for the involvement of spinal fractalkine receptor in pain facilitation and spinal glial activation in rat model of monoarthritis. Pain 129, 64–75.

Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14.

Ting, E., Guerrero, A.T., Cunha, T.M., Verri Jr., W.A., Taylor, S.M., Woodruff, T.M., Cunha, F.Q., Ferreira, S.H., 2008. Role of complement C5a in mechanical inflammatory hypernociception: potential use of C5a receptor antagonists to control inflammatory pain. Br. J. Pharmacol. 153, 1043–1053.

Torres, R., Macdonald, L., Croll, S.D., Reinhardt, J., Dore, A., Stevens, S., Hylton, D.M., Rudge, J.S., Liu-Bryan, R., Terkeltaub, R.A., Yancopoulos, G.D., Murphy, A.J., 2009. Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis. Ann. Rheum. Dis. 68, 1602–1608.

Verri Jr., W.A., Cunha, T.M., Parada, C.A., Poole, S., Cunha, F.Q., Ferreira, S.H., 2006. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacol. Ther. 112, 116–138.

Verri Jr., W.A., Guerrero, A.T., Fukada, S.Y., Valerio, D.A., Cunha, T.M., Xu, D., Ferreira, S.H., Liew, F.Y., Cunha, F.Q., 2008. IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. Proc. Natl. Acad. Sci. U. S. A. 105, 2723–2728.