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CXCR2 is critical for bacterial control and development of joint damage and pain in *Staphylococcus aureus*-induced septic arthritis in mouse

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Staphylococcus aureus is the main pathogen associated with septic arthritis. Upon infection, neutrophils are quickly recruited to the joint by different chemoattractants, especially CXCR1/2 binding chemokines. Although their excessive accumulation is associated with intense pain and permanent articular damage, neutrophils have an important function in controlling bacterial burden. This work aimed to study the role of CXCR2 in the control of infection, hypernociception and tissue damage in S. aureus-induced septic arthritis in mice. The kinetics of neutrophil recruitment correlated with the bacterial load recovered from inflamed joint after intra-articular injection of S. aureus. Treatment of mice from the start of infection with the non-competitive antagonist of CXCR1/2, DF2156A, reduced neutrophil accumulation, cytokine production in the tissue, joint hypernociception and articular damage. However, early DF2156A treatment increased the bacterial load locally. CXCR2 was important for neutrophil activation and clearance of bacteria *in vitro* and *in vivo*. Start of treatment with DF2156A 3 days after infection prevented increase in bacterial load and reduced the hypernociception in the following days, but did not improve tissue damage. In conclusion, treatment with DF2156A seems be effective in controlling

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tissue inflammation and dysfunction but its effects are highly dependent on the timing of the treatment start.

Keywords: Chemokine · CXCR2 · Neutrophil · Septic arthritis · *Staphylococcus aureus*

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Introduction

Septic arthritis is an infectious articular disease with an annual incidence of 6–12 cases per 100 000 habitants and associated with high morbidity and mortality [1, 2]. Different microorganisms, predominantly bacteria, can colonize the joint cavity and cause disease. The gram positive cocci *Staphylococcus aureus* are responsible for about 60% of septic arthritis cases [3, 4]. The local clinical signs of the disease include redness, edema and painful joints with limited movement and fever [5]. The articular damage is an important feature and a challenge, as about 25–50% of patients have irreversible articular damage with total loss of joint function [6].

The presence of the microorganism in the joint elicits rapid activation of resident cells through the recognition of pathogen-associated molecular patterns by innate immune receptors that lead the release of several inflammatory mediators [7]. Neutrophils are major contributors for bacterial clearance [8]. Different neutrophil-related chemoattractants, including leukotriene B4 [9], the complement component C5a [10] and chemokines [11] are produced and guide the massive recruitment of neutrophils to the joint.

Chemokines are small proteins that bind to G protein-coupled receptors (GPCRs) and attract and activate cells [12]. Here, our focus was on the role of CXCR1 and CXCR2 for *S. aureus*-induced inflammation. CXCR1 and CXCR2 were the first members of the chemokine receptor family to be cloned and share a high degree of homology [13]. Chemokines that bind CXCR1 or CXCR2 share a common ELR^+ motif in their structure. Although mice possess both receptors [14–16], they only have a few homologues for the seven human ELR^+ CXC chemokines and the function of murine CXCR1 is still unclear [17]. Moreover, human and mouse ELR^+ CXC chemokines vary in activity according to their producer cells, receptor affinity and specificity [18]. The compound used in our study is shown to inhibit both CXCR1 and CXCR2 [19].

Once activated, neutrophils express high levels of CXCR1 and CXCR2 on their surface [20]. In the tissue, neutrophils control *S. aureus* infection by their phagocytic capacity [21]. Their machinery to kill includes the production of reactive oxygen species (ROS) [22], neutrophil extracellular traps (NETs) [23] and antimicrobial peptides and lytic enzymes stored in specific granules [24]. However, the presence of neutrophils is frequently associated to tissue damage and pain. Tissue damage and pain positively correlate to neutrophil numbers, state of neutrophil activation and their persistence in the tissue [25]. In sterile inflammation, the pharmacological blockade of neutrophil migration to the tissue may avoid or decrease tissue damage and dysfunction [26, 27]. Accordingly, we previously demonstrated that the blockade of CXCR1/2 prevented excessive joint inflammation and hypernociception in a model of antigen-induced arthritis in mice [28, 29]. However, the benefit of the blockade of neutrophil recruitment in infectious diseases is less clear. During infection, it is important to fine-tune the activation of the immune system to induce bacterial clearance and to avoid excessive inflammation-induced pain and joint damage. Here, we showed that the presence of neutrophils in the joint following *S. aureus* is associated with the number of bacteria, pain, and tissue damage. The blockade of CXCR1/2 from the beginning of infection was effective to control joint hypernociception and damage, although it increased bacterial load locally. However, blockade of these receptors later in the course of infection improved articular pain, but did not influence the number of bacteria.

Results

A single joint injection with *Staphylococcus aureus* causes prolonged inflammation and tissue damage

Septic arthritis caused by *S. aureus* infection is characterized by massive influx of cells, mainly neutrophils into the affected joint [30]. Here, a single injection of *S. aureus* into the tibiofemoral joint of mice promoted intense accumulation of cells into the joint cavity at 24 hours. This was sustained up to day 14 and then the total number of leukocytes started to decrease. Significant number of leukocytes remained present in the joint even at day 28 (Fig. 1A). Neutrophils were the major cell type along all evaluated time points (> 75% until day 7) and neutrophil kinetics followed a similar profile as the total leukocyte numbers (Fig. 1A and B). The number of mononuclear cells increased later, peaking at day 14 and decreasing thereafter (Fig. 1C). The number of bacteria recovered from the infected joint was highest at day 1 and decreased thereafter. Of note, even at day 28 after infection there was still a significant number of bacteria in the joint (Fig. 1D). Overall, there was a good association between the presence of neutrophils and bacteria in the joint.

Pain and permanent joint damage are critical consequences in patients that develop bacterial septic arthritis [31]. Histopathological damage peaked at day 7 after injection (Fig. 2A and B). The first day of infection was characterized by a marked influx

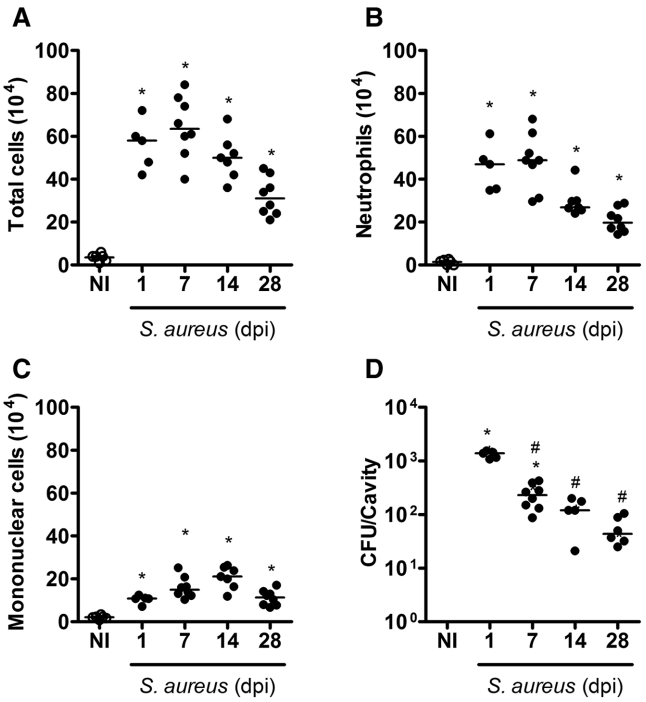

Figure 1. Kinetics of cell recruitment and bacterial load in the joint. Mice were injected intra-articularly with *S. aureus*. Cells were harvested from the cavity 1, 7, 14 or 28 days after injection. (A) The total number of leukocytes, (B) neutrophils and (C) mononuclear cells recruited to the joint were determined. The joint was removed and the bacterial load (D) was evaluated after the same infection periods. Data are shown as median, representative of three independent experiments with 30 mice per experiment. $^{*}p<0.05$ or $^{\#}p<0.01$ when compared with the NI (non-infected) group (ANOVA test followed by Newman Keuls' test). $N = 5$–8 mice per group.

of leukocytes, especially neutrophils. Intense cell infiltration persisted throughout the observation period, but there was a remarkable presence of synovial hyperplasia and bone reabsorption at later periods (Fig. 2A and B). We evaluated the density of proteoglycans, important constituents of cartilage. Corroborating with the histopathological score, there was significant loss of proteoglycans throughout the observation period, with the most abundant loss at day 7 after infection (Fig. 2C). Joint dysfunction, as assessed by measuring hypernociception, was present throughout the observation period (Fig. 2D). Thus, a single injection of *S. aureus* caused longstanding joint inflammation accompanied by significant tissue damage and pain. Since most of the evaluated parameters peaked at day 7 after infection, this time point was chosen for most subsequent experiments.

CXCR1/2 blockade reduces neutrophil influx and ameliorates tissue inflammation and hypernociception

It is well established that excessive and prolonged presence of activated neutrophils in the joint may cause and amplify local inflammation, tissue damage and pain and that the blockade of CXCR2 efficiently controls these changes in non-infectious arthritis [32, 33]. We used the compound DF2156A, a non-competitive antagonist of CXCR1 and CXCR2, to inhibit the infiltration and activation of neutrophils in our study. In the first set of experiments, a group of mice was treated with DF2156A 1 hour before the injection of *S. aureus* and this treatment was repeated daily for the 6 subsequent days. There was a reduction in the number of total leukocytes accumulated in the joints of DF2156A-treated mice as compared to vehicle-treated control mice (Fig. 3A). Importantly, there was partial but not complete blockade of neutrophil influx into the joint (Fig. 3B). Likewise the myeloperoxidase (MPO) activity (Fig. 3C) was significantly reduced. There was no decrease in mononuclear cell numbers recovered from the joint cavity (Fig. 3D). Production of the neutrophil attractant CXCL1 was significantly reduced in inflamed tissue (Fig. 3E). In addition, treatment with DF2156A led to lower concentrations of TNF-α and IL-1β, comparable to levels found in uninfected mice (Fig. 3F and G).

Treatment with DF2156A also led to reduced hypernociception, as evidenced by an increase of the withdrawal threshold in the flexed joint (Fig. 4A). As prostaglandins play a major role in pain development [34], we checked for COX-2 expression, the major enzyme responsible for the synthesis of prostaglandins. The treatment with DF2156A significantly decreased COX-2 expression (Fig. 4B). In addition, this treatment efficiently reduced tissue (Fig. 4C) and cartilage (Fig. 4D) damage. Taken together, these results suggest that the control of neutrophil migration to the joint from the beginning of the infection decreased *S. aureus*-induced inflammation and preserved joint integrity.

CXCR1 and CXCR2 are important for the activation and clearance of bacteria by neutrophils

Since neutrophils have a fundamental role in controlling the bacterial load in various models of bacterial infection, we investigated whether the treatment with DF2156A could affect the clearance of *S. aureus* from the joint. As seen in Fig. 5A, the treatment with DF2156A from the very beginning of the infection impaired bacterial clearance. As indicated above, early treatment with DF2156A reduced the accumulation of neutrophils into the joint (Fig. 3B).

As shown in Fig. 1, there was a significant neutrophil influx at day 7. To evaluate whether constant activation of neutrophils by chemokines acting on CXCR1/2 was necessary for controlling bacterial replication, mice received local treatment with DF2156A at day 7. Figure 5B shows that local treatment with DF2156A impaired bacterial clearance. To confirm the importance of CXCR1/2 for clearance of the *S. aureus* strain used in this study, we incubated human neutrophils with *S. aureus* in the presence or absence of different concentrations of CXCL8, a ligand for CXCR1/2. Neutrophils alone partially control bacterial growth, but the presence of CXCL8 enhanced bacterial killing (Fig. 5C and D). CXCL8 alone, without neutrophils, had no effect on the bacteria and DF2156A neutralized the effect of CXCL8 (Fig. 5D). Altogether, these results suggest that CXCR1/2 receptors drive neutrophil migration and activation and are necessary for the murine host to deal with *S. aureus* infection.

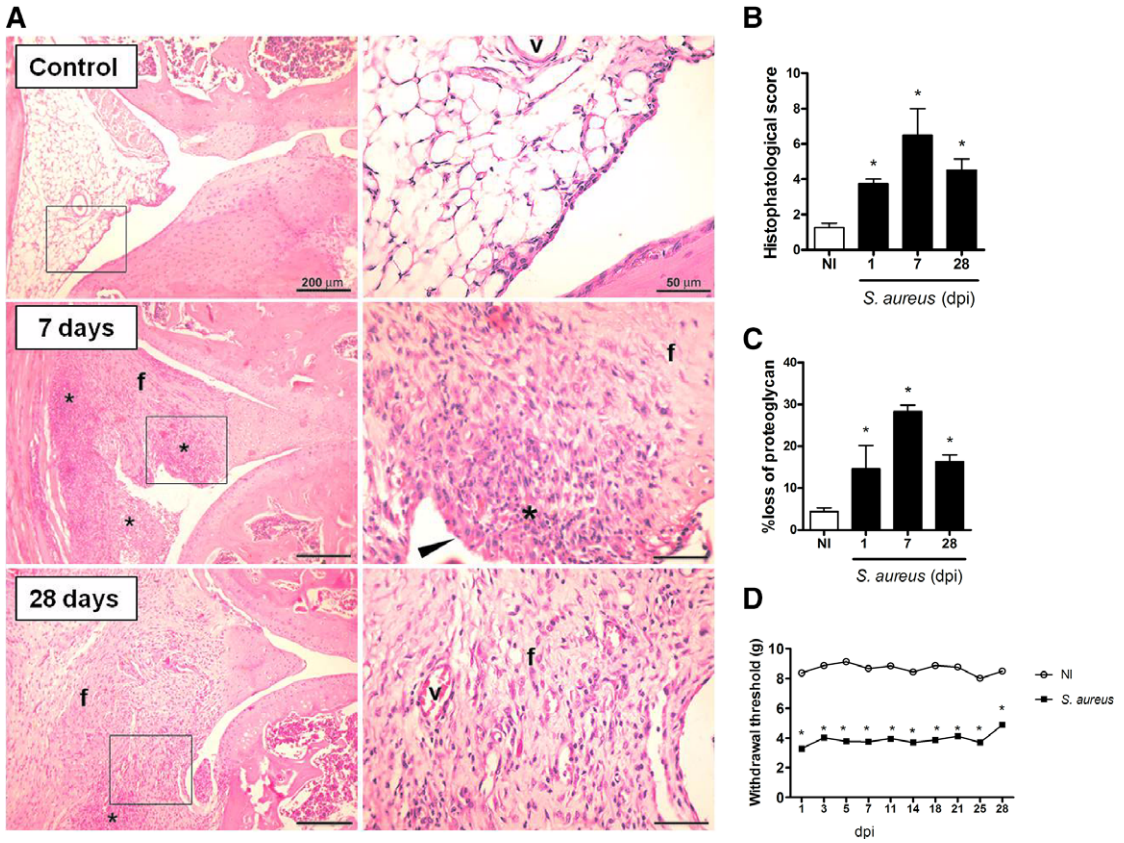

Figure 2. Kinetics of articular damage and hypernociception in *S. aureus*-induced arthritis. The joint was removed 1, 7, or 28 days after *S. aureus* infection and tissues processed for histopathological analyses. (A) Representative images of joints (v - blood vessels; f - collagen fibers; * - cellular infiltrate; arrowhead - synovial hyperplasia). Scale bar: 200 or 50 μm, as reported in figure. (B) Histopathological score and (C) % loss of proteoglycans were determined. (D) Hypernociception was evaluated using an electronic analgesimeter. Data are shown as mean ± SEM from one representative out of two independent experiments with 25 mice per experiment. dpi – days post infection. * $p<0.05$ when compared to the non-infected (NI) group (ANOVA test followed by Newman Keuls' test). $N = 3–5$ mice per group.

Delayed DF2156A treatment prevents increased bacterial load and limits joint hypernociception

The time elapsed between infection and the first medication in septic arthritic patients is critical for disease progression [31]. We showed that treatment with DF2156A from the beginning of the infection could prevent most clinical parameters. Next, we started the treatment with DF2156A 3 days after infection. As seen in Fig. 2, joint dysfunction is observed very early during infection, suggesting that this therapeutic schedule (2 days after onset of symptoms) would be therapeutically relevant. Delayed treatment with DF2156A (from day 3 after infection) also prevented the excessive accumulation of neutrophils in the joint at days 4 and 7 after infection, as compared to vehicle-treated control mice (Table 1). Importantly, delayed treatment with DF2156A did not result in increased bacterial load (Fig. 6A–C). Delayed treatment decreased joint pain early in the course of infection (day 4) but not at day 7 after infection (Fig. 6D–F). There was no reduction of articular damage as assessed by histology (Table 1).

Discussion

Different forms of joint inflammation are accompanied by permanent pain and tissue damage, conditions that cause severe disabilities in patients. It is well established that excessive and constant recruitment of leukocytes to the affected joint is critical to cause these events. However, with respect to septic arthritis, the cellular recruitment to the joint is fundamental for the control of infection. Thus, fine-tuning between cellular migration and activation to eliminate the microorganism and prevention of excessive tissue damage must be aimed at. In this study, we investigated the role of CXCR2 in the recruitment and activation of neutrophils in a model of septic arthritis. Our main findings can be summarized as follows: (i) A single injection of *S. aureus* into the joint of mice caused prolonged joint inflammation, tissue damage and hypernociception, that was associated with excessive accumulation of neutrophils into the joint; (ii) The systemic blockade of CXCR2 from the beginning of the infection decreased tissue inflammation, pain and damage, but led to an increase in bacterial load; (iii) CXCR2 activation is very important for the control of *S. aureus*

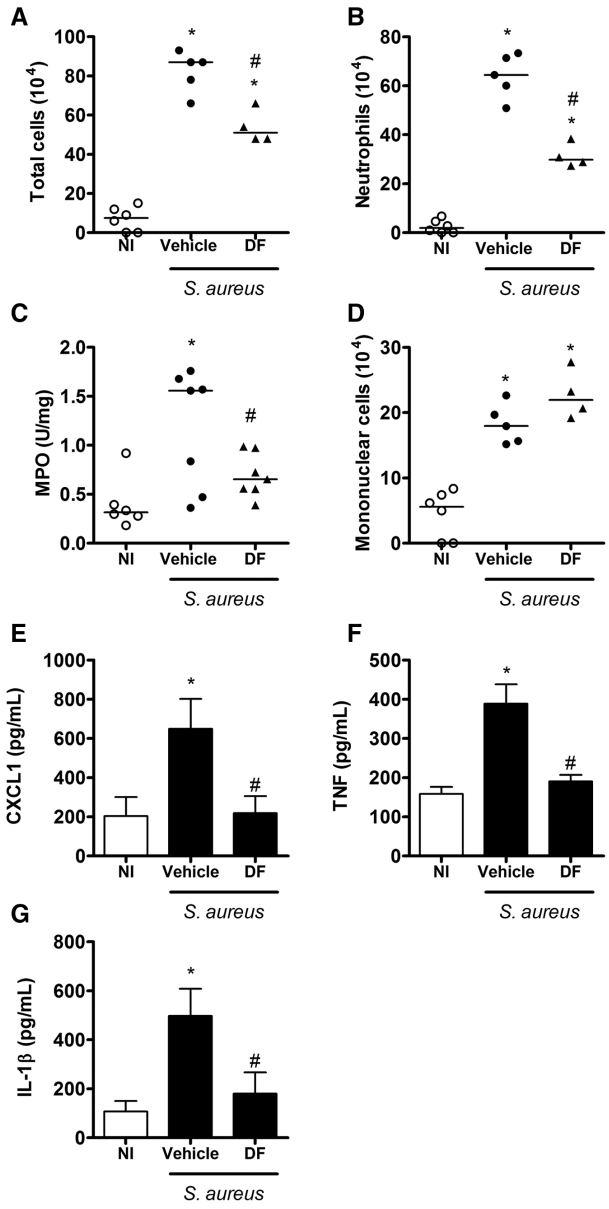

Figure 3. The blockade of CXCR1/2 decreased neutrophil accumulation and the production of pro-inflammatory mediators in *S. aureus*-infected joints. Mice were infected with *S. aureus* into the tibiofemoral joint and evaluated 7 days later. A group of mice were treated with DF2156A 1 h prior to the injection of *S. aureus* and daily for the following 6 days and (A) the total number of leukocytes, (B) neutrophils and (D) mononuclear cells evaluated. The inflamed periarticular tissue was processed for the quantification of MPO activity (C) CXCL1, (E) TNF (F) and (G) IL-1β protein by ELISA. Data are shown as median or mean ± SEM from one representative of three independent experiments with 25 mice per experiment. $^{*}p<0.05$ when compared with the NI group; $^{\#}p<0.05$ when compared to the vehicle-treated infected group (ANOVA test followed by Newman Keuls' test). N = 4-7 mice per group.

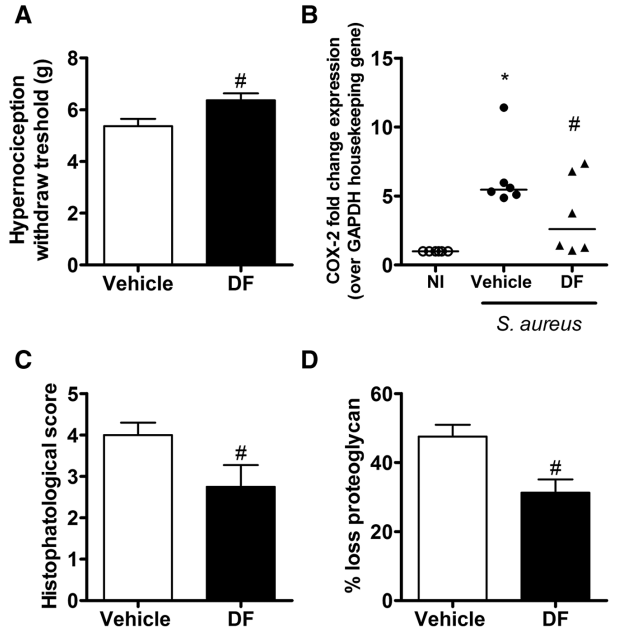

Figure 4. The blockade of CXCR1/2 decreased hypernociception and tissue damage in *S. aureus*-infected joint. Mice were infected with *S. aureus* into the tibiofemoral joint and evaluated 7 days later. A group of mice was treated with DF2156A 1 h prior the injection of *S. aureus* and daily for the following 6 days. (A) The intensity of hypernociception was evaluated as the paw withdrawal threshold. The periarticular tissue was removed and expression of COX-2 (B) determined by PCR and normalized using GAPDH gene. Whole joints were removed and processed for (C) histopathology and (D) the analysis of the loss of proteoglycan. Data are shown as median or mean ± SEM, represent one out of two independent experiments with 15 mice per experiment. $^{*}p< 0.05$ when compared with the NI group (ANOVA test followed by Newman Keuls test). $^{\#}p<0.05$ when compared to the vehicle-treated infected group (ANOVA test followed by Newman Keuls' test – histology and COX-2; or *t*-test followed by unpaired test - hypernociception). N = 3–10 mice per group.

infection by neutrophils into the joint; (iv) The blockade of CXCR2 from day 3 after infection was still effective to decrease hypernociception but did not influence the bacterial load in the joint nor tissue damage.

Neutrophils are the first cell type recruited to the tissue during bacterial infection and have a potent machinery to control these microorganisms [35]. In an experimental model of septic arthritis induced by intravenous injection of *S. aureus*, joint swelling and erythema in mice limbs were strictly dependent on the presence of neutrophils in the tissue [36]. The bacterial strain used here did not cause arthritis if injected intravenously in immunocompetent animals (data not shown). On the other hand, the local injection of our *S. aureus* strain was sufficient to provoke longstanding accumulation of neutrophils in the synovial cavity. Of interest, the number of bacteria in the joint followed similar kinetics to the number of neutrophils, suggesting neutrophils were relevant to control bacterial infection. Indeed, it has been shown that the depletion of neutrophils with anti-LY6G antibody caused high mortality due to bacterial spread to the circulation [37].

Chemokines that bind to CXCR1 or CXCR2 are potent chemoattractants and activators of neutrophils [40]. Here, the systemic treatment with DF2156A, a non-competitive antagonist of CXCR1 and CXCR2, started before the infection did not abolish the recruitment of neutrophils, but was sufficient to increase the bacterial load in the joint. Nevertheless, we did not detect bacteremia in DF2156A-treated mice (data not shown). Our data corroborate with some publications in which human neutrophils improved the

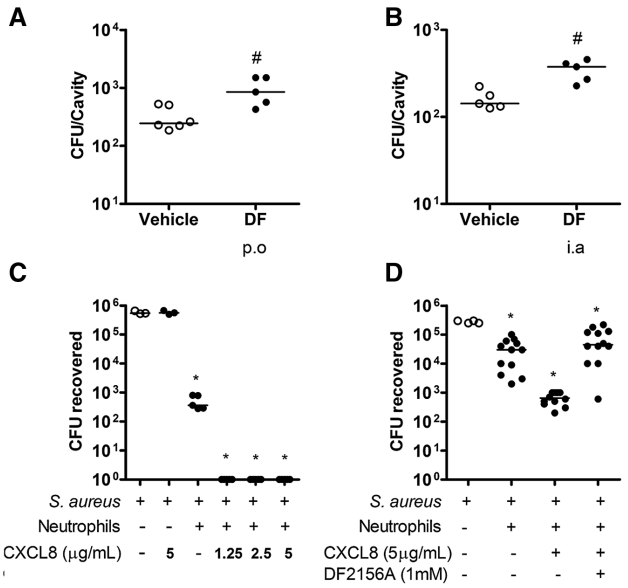

Figure 5. CXCR1/2 are important for the control of *S. aureus*. Mice were infected with *S. aureus* into the tibiofemoral joint and evaluated 7 days later. The inflamed periarticular tissue was collected for the analysis of the bacterial load. (A) A group of mice were treated with DF2156A 1 h prior the injection of *S. aureus* and daily for the following 6 days. (B) In another experimental approach, a group of mice was treated locally with DF2156A from day 3 to day 6 after infection. (C, D) Peripheral blood human neutrophils were incubated with different concentrations of CXCL8 or DF2145A and infected with *S. aureus* at a MOI of 10:1 (bacteria:cell) for 3 h for the analysis of bacterial killing. Data shown as median from one representative out of three independent experiments with 36 mice per experiment for panels A and B. Data in panel C were confirmed in two different laboratories [at UFMG (C) and KU Leuven (D)]. $^{\#}p<0.01$ when compared to the vehicle-treated infected group (*t*-test followed by unpaired test) or $^{*}p<0.01$ when *S. aureus* cultivated without neutrophils compared CXCL8 or DF2156A (ANOVA test followed by Newman Keuls test). $N = 5$–6 mice per group.

efficacy to kill *Pseudomonas aeruginosa* [38] and *Candida albicans* [39] by CXCR1/2 activation. To check for a direct role of CXCR1/2 in the activation of neutrophils to control *S. aureus* infection, we demonstrated that the local treatment with DF2156A increased the bacterial load in the joint. Furthermore, human neutrophils increased their killing capacity in the presence of CXCL8 an effect which was neutralized by the CXCR1/2 antagonist.

The involvement of neutrophils in infectious diseases encompasses dual characteristics. They possess the machinery to control microorganisms but, as a side effect this may cause important tissue damage [40]. Once activated, neutrophils secrete granules, enzymes, reactive oxygen species and some antimicrobial peptides that can damage the tissue [41, 42]. Furthermore, the amplification of tissue inflammation is accompanied by an increase in cytokine production, e.g. TNF and IL-1. The blockade of those cytokines is beneficial to reduce tissue damage, although it may potentially harm the clearance of infections [43, 44]. In this context, the absence of IL-1 and its receptors impairs bacterial elimination in septic arthritis [45]. However, the presence of *S. aureus per se* can produce and release bacterial enzymes and virulence factors that directly lead to tissue damage [46, 47]. Thus, the elicited inflammation following an infection must be well controlled to avoid irreversible joint damage and dysfunction. The presence of neutrophils at the onset of bacterial infection is very important for the initial control of infection, avoiding bacterial spread [48]. Clinically, an eventual attenuation/blockade of neutrophil activation or migration would not occur immediately after infection, i.e. before the clinical signs of infection-elicited inflammation. In this context, we started the treatment with DF2156A from the 3rd day after infection. With such treatment, there was no increase in bacterial colonies in the joint from the 4th to 7th day after infection when compared to non-treated mice. Thus, the permission of neutrophil influx to the joint since the first signs after *S. aureus* infection could be sufficient to control an excessive presence of microorganisms in the tissue. However, this was not sufficient to prevent tissue damage.

Pain is a critical symptom in septic arthritis patients [49]. Several clinical observations and laboratory experiments point out that neutrophils have a direct involvement in joint pain under different stimuli and diseases by the production of several algogenic mediators, including prostaglandins and cytokines [50, 51]. On the other hand, CXCR1/2 and their ligands also contribute to pain by direct activation of afferent nociceptive fibers [52]. Interestingly, a study showed that *S. aureus* can directly trigger action potentials in nociceptive neurons through N-formylated peptides and α-haemolysin toxin [53]. In our study, a single injection of *S. aureus* caused persistent hypernociception up to 28 days after the infection and the blockade of CXCR1/2, even started 3 days after infection, reduced mechanical hypernociception. Moreover, treatment of infected mice with the CXCR1/2 inhibitor reduced COX-2 expression induced by the infection. Thus, the reduction of joint hypernociception in our model seems to be more dependent on CXCR1/2 and neutrophils than on the presence of *S. aureus* alone; i.e. *S. aureus* is necessary to trigger the cascade of events leading to joint dysfunction but the bacterium is not sufficient to trigger dysfunction on its own.

Patients that develop septic arthritis can have serious articular damage even with appropriate treatment. About 25–50% of patients have permanent dysfunction of the affected joint [54].

Table 1. The blockage of CXCR2 in a late stage did not decrease the articular damage

	4 dpi		7 dpi	
Groups	*S. aureus*	*S. aureus* + DF2156A	*S. aureus*	*S. aureus* + DF2156A
Histopathological score	8.6 ± 0.25	7.0 ± 0.81	2.8 ± 0.52	4.1 ± 0.28

Mice were treated with DF2156A 3 days after *S. aureus* injection and tissue damage evaluated at days 4 and 7. Data are shown as mean ± SEM, representative of three independent experiments. $n = 5$ mice per group.

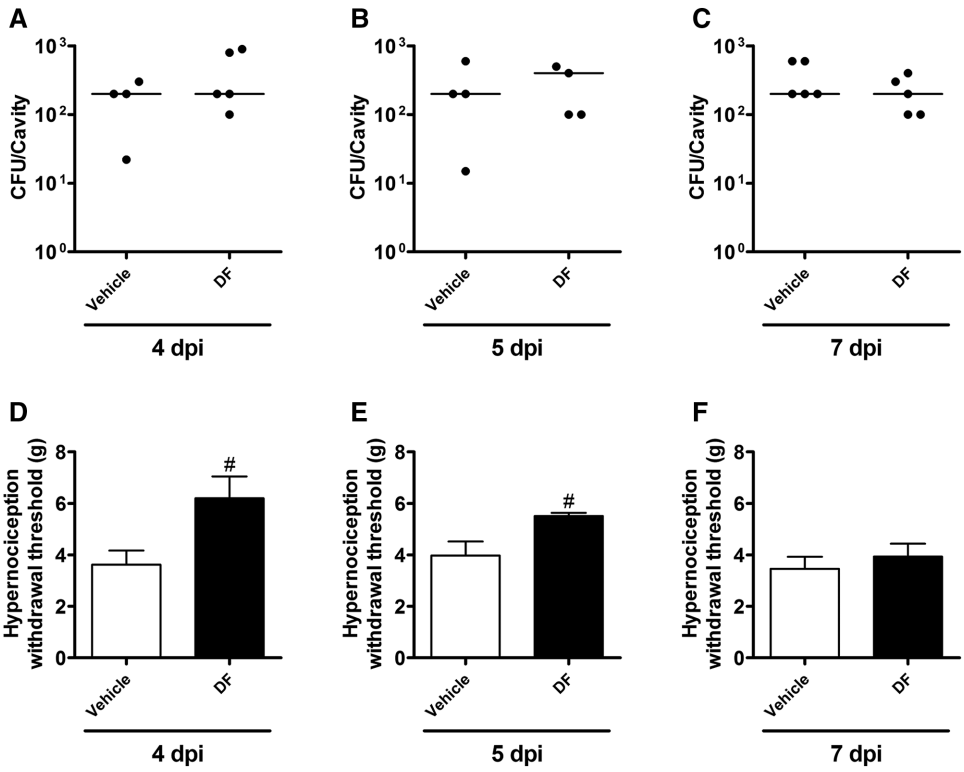

Figure 6. The blockage of CXCR1/2 in a late stage decreases hypernociception and prevents the increase in bacterial load. Mice were infected with *S. aureus* into the tibiofemoral joint and treatment was started 3 days after infection (dpi). Different groups of mice were treated daily with DF2156A or vehicle. The intensity of hypernociception was evaluated as the paw withdrawal threshold at days 4 (A), 5 (B) and 7 (C) post infection. The inflamed periarticular tissue was collected for the analysis of the bacterial load at the same time points (D, E and F). Data are shown as mean ± SEM, from a representative experiment. $^{\#}p<0.01$ when compared to the vehicle-treated infected group (t-test followed by unpaired test). $N = 4–5$ mice per group.

Experimentally, several studies have demonstrated that the blockade of CXCR1/2 receptors or their ligands are beneficial to the control of inflammation, tissue damage and dysfunction, mainly in non-infectious conditions [55, 56]. Our current study shows that accumulation of neutrophils in the joint in a CXCR1/2-dependent manner is directly associated with tissue damage. However, blockade of CXCR1/2 is effective to prevent tissue damage only if the treatment with the CXCR1/2 antagonist is initiated early in the course of infection. The delayed treatment was not able to prevent tissue damage, showing that the early neutrophil influx and CXCR1/2 activation during the first 3 days of infection were enough to cause joint damage in this model. In patients, a delay in starting treatment with antibiotics and anti-inflammatory compounds cannot prevent tissue damage [57, 58]. Experimentally, the combined therapy of antibiotics with anti-TNF [59] or corticosteroids and bisphosphonate [60] were effective to decrease bone resorption and tissue damage in *S. aureus*-induced arthritis. However, the *S. aureus* strain used here is extremely susceptible to Vancomycin, the main antibiotic used clinically for *S. aureus*-induced arthritis. Mice treated with Vancomycin only, had all inflammatory makers abrogated after *S. aureus* infection (data not shown), making the combined treatment irrelevant.

In conclusion, CXCR1/2 receptors contribute to control *S. aureus* replication in the context of septic arthritis. In addition, neutrophils also have a major role in driving joint damage and dysfunction. The blockade of CXCR1/2 seems be effective in controlling tissue inflammation and dysfunction when started early in the context of infection but has an intrinsic risk of worsening infection in treated individuals. It is necessary that future studies examine the potential benefit of the administration of CXCR1/2 antagonists in individuals treated with antibiotics.

Materials and methods

Mice and reagents

Eight-to-ten-week-old male C57BL/6J mice (375 in total) were purchased from the Centro de Bioterismo of the Universidade Federal de Minas Gerais. All animals were maintained with filtered water and food *ad libitum* and kept in a controlled environment. Experiments received prior approval by the animal ethics committee of the UFMG (CEUA 236/2012). The non-competitive allosteric inhibitor DF2156A was kindly provided by Dompé Pharma - Italy. Full length CXCL8 (containing 77 amino acids) was purchased from R&D Systems or Peprotech and Histopaque-1119 and Histopaque-1077 were obtained from Sigma.

Experimental model of septic arthritis

Staphylococcus aureus ATCC 6538 was grown in brain heart infusion agar (BHI) supplemented with 5% sheep blood for 24 hours at 37°C. The bacterial solution was prepared in PBS at a concentration of 10^7 CFU/mL. Ten microliters of the solution were injected into the tibiofemoral knee joint of mice placed under anesthesia (60:5mg/kg ketamine:xylazine injected intraperitoneally). Viable counts were used to check the concentration of injected bacteria. Inflammatory parameters and bacterial load were evaluated at different time points after bacterial injection (1, 4, 5, 7 and 28 days). In a different set of experiments the mice were treated with DF 2156A by gavage (10 mg/kg diluted in carboxymethyl cellulose) or by local injection (10uM) [19]. Groups of mice were culled for cervical dislocation and the articular cavity was washed with phosphate buffered saline (PBS) – 3% bovine serum albumin for cell counts. The number of leukocytes from the articular cavity was determined in a Neubauer chamber, after staining the cells with Turk's solution. Differential counts were performed on Cytospin (Shandon III) preparations by evaluating the percentage of each leukocyte type on a slide stained with May-Grunwald-Giemsa. Periarticular tissue was removed from the joints for evaluation of cytokine and chemokine production. The inflamed joint was removed, homogenized and placed in brain heart infusion (BHI) agar supplemented with blood for 24 h at 37°C to check the bacterial load.

Measurement of chemokine, cytokines, and myeloperoxidase

Periarticular tissue was collected and homogenized in PBS containing protease inhibitors [28]. Samples were processed and the supernatant was evaluated by ELISA for cytokine and chemokine concentrations, in accordance with the manufacturer's instructions (R&D Systems). The pellet was used for MPO activity assay by measuring the change in OD at 450 nm using tetramethylbenzidine [28].

Evaluation of hypernociception

Evaluation of mechanical hypernociception was performed as previously described [29], using an electronic pressure meter (INSIGHT Instruments, Brazil). The flexion-elicited withdrawal threshold was used to infer behavioral responses associated with pain. Results are expressed as the change in withdrawal threshold (in grams).

Histopathologic analysis

The whole tibiofemoral joints were fixed in 10% buffered formalin (pH 7.4), decalcified for 30 days in 14% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Two sections of knee joints were microscopically examined by a single pathologist, and scored in a blinded manner. The histologic score was adapted from an arthritis index as described previously [61]. The parameters evaluated were: severity of synovial hyperplasia, intensity and extension of inflammatory infiltrate, vascular hyperemia, presence of inflammatory cells in the synovial cavity and changes in tissue architecture. These criteria result in a maximal score of 9 points.

Cell culture and killing

Neutrophils were isolated from blood of healthy donors using a Histopaque gradient. Neutrophils were incubated with CXCL8, CXCL8/DF2156A or RPMI medium for 30 min and then *S. aureus* was added in a multiplicity of infection (MOI) of 10:1 (bacteria:cell) for 3 h. The number of surviving bacteria was determined by incubation of the cells with 1% Triton X-100 for 10 min to lyse them. Subsequently, serial dilutions were prepared and incubated on agar plates overnight at 37°C. Bacterial colonies were counted and expressed in CFU recovered.

Real time PCR

Total RNA was isolated from synovial tissue using Trizol reagent (Ambion, Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA). Real-time PCR quantitative mRNA analyses were performed on a 7500 Fast Real-Time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) after reverse transcription of 1 μg RNA using SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Thermo Fisher Scientific). The relative level of gene expression was determined by the comparative threshold cycle method, as described by the manufacturer, whereby data for each sample were normalized to a GAPDH constitutive gene and expressed as a fold change compared with control. The following primer pairs were used: for *gapdh*, 5′-ACG GCC GCA TCT TCT TGT GCA-3′ (forward) and 5′-CGG CCA AAT CCG TTC ACA CCG A-3′ (reverse); for COX-2 5′-ACACCTTCAACATTGAAGACC-3′ (forward) and 5′ ATCCCTTCACTAAATGCCCTC-3′ (reverse).

Statistical analyses

Data were expressed as mean ± standard of the mean (SEM) and analysis performed using the statistical software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Differences between means were evaluated using analysis of variance (ANOVA test), followed by Newman-Keuls and *t*-test followed by unpaired test. Results with $p<0.05$ were considered significant.

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