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CONTROL OF INFECTION VERSUS ARTICULAR DAMAGE: ROLE OF NEUTROPHILS AND 5- LIPOXYGENASE IN SEPTIC ARTHRITIS

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LIST OF ABBREVIATIONS

AA: arachidonic acid

ACKR: atypical chemokine receptor

BHI: brain heart infusion

cAMP: Cyclic adenosine monophosphate

CD: cluster of differentiation

CFU: colony-forming units

CpG: cytosine- phosphate- guanine

DC: dendritic cell

DMARDs: disease-modifying anti-rheumatic drugs

DNA: Deoxyribonucleic acid

Dpi: days post infections

DT: diphtheria toxin

DTR: diphtheria toxin receptor

EDTA: Ethylenediamine tetraacetic acid

ELR motif: glutamic acid-leucine-arginine

FPR: formyl peptide receptors

GAG: glycosaminoglycan

GPCR: G protein–coupled receptors

HIV: human immunodeficiency virus

IL- : Interleukin-

i.p.: intraperitoneal

i.v.: intravenous

LO: lipoxygenase

LTB: leukotriene

LXA: lipoxin

MOI: multiplicity of infection

MPO: myeloperoxidase

MRI: Magnetic resonance imaging

mRNA: messenger Ribonucleic acid

MRSA: methicillin-resistant *Staphylococcus aureus*

NADPH: Nicotinamide adenine dinucleotide phosphate oxidase

NET: neutrophil extracellular traps

NI: non-infected

NK: natural killer

PAMP: Pathogen-associated molecular pattern

PCR: polymerase chain reaction

PGN: peptidoglycan

RA: rheumatoid arthritis

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute

TLR: Toll-like receptors

TNF: tumor necrosis factor

WT: wild type

FIGURE LIST

TABLE LIST

ABSTRACT

Staphylococcus aureus is the main pathogen associated with septic arthritis. Upon infection, neutrophils are quickly recruited to the joint by different chemoattractants, such as chemokines and leukotriene B_4 (LTB_4). 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 chemokines and the enzyme 5-lipoxygenase (5-LO) in the control of infection, hypernociception, and tissue damage in *S. aureus*-induced septic arthritis in mice. The blockade of the chemokine receptors CXCR1/2 from the beginning of infection decreased neutrophil accumulation in *S. aureus*-infected joint, contributing to the reduction of articular damage and hypernociception, although it increased the bacterial load. CXCR1/2 was important for the killing of *S. aureus* by purified human neutrophils. The later start of the treatment did not increase the bacterial load, but only transiently decreased hypernociception and did not improved tissue damage. Using another strategy, we blocked the chemokine binding to glycosaminoglycans (GAGs) by intravenously injection of CXCL9 (74-103). Although this treatment decreased neutrophil accumulation in peptidoglycan- and *S. aureus*-infected joints, during infection it was not effective to reduce articular damage and hypernociception and increased the bacterial load. In another set of experiments, 5-lipoxygenase $(LO)^{-/-}$ mice had a reduced neutrophil accumulation, joint damage, hypernociception, and bacterial load 7 days after *S. aureus* injection compared to wild type (WT) mice. At day 4, there were increased numbers of activated cluster of differentiation 11c $(CD11c)^{+}$ cells and T lymphocytes in the joint and draining lymph node of $5\text{-}LO^{-/-}$ compared to WT mice. At this time point, there was an increase of lipoxin A_4 (LXA_4)/LTB_4 ratio in the joint, two byproducts from 5-LO activity. The blockade of the LXA_4 surface receptor, ALX/formyl peptide receptor (FPR)2, in WT mice decreased the bacterial load in infected joints. Corroborating, the injection of LXA_4 in $5\text{-}LO^{-/-}$ mice increased the number of recovered bacteria. Interestingly, LXA_4-treated human dendritic cells decreased chemotaxis under CCL21 stimulation. In conclusion, the blockade of CXCR1/2, chemokine-GAG binding and LXA_4 activity could be useful for the improvement of current treatment of *S. aureus*-induced arthritis by different mechanisms, but commonly by decreasing neutrophil recruitment.

Key words: arthritis, *Staphylococcus aureus*, chemokines, 5-lipoxygenase, lipoxin A4

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1. INTRODUCTION

1.1 Septic arthritis

Septic arthritis is an infectious articular disease associated with high morbidity and mortality among the patients. The annual incidence in developed countries is 6-12 cases per 100,000 habitants (1–4). Bacteria, viruses, fungi and protozoa may invade joints and produce injury. However gram positive bacteria, especially *Staphylococcus aureus*, are the main microorganisms that cause septic arthritis (5). Invasion of bacteria into the synovial space can occur predominantly by 2 routes: either through a hematogenous spread (most common) or by direct invasion (6). The synovium is extremely vascular and contains no limiting basement membrane, facilitating the access to the synovial space. Thus, bacteria may spread directly from adjacent osteomyelitis or from a local soft-tissue infection and could reach the joint during diagnostic or therapeutic procedures, penetrating trauma, prosthetic surgery and less common animal bites (7,8).

Septic arthritic patients present with a combination of single swollen, warm and painful joints with a decrease in range of motion in the infected joint. Fever is present only in 30-40% of the cases (9). Normally a single synovial joint is affected such as the knee, hip, ankle or elbow. The hip is the more frequently affected joint in children. Atypical joint infection, including the sternoclavicular, costochondral and sacroiliac joint may be common in intravenous drug users (10). Polyarticular septic arthritis is not common and usually accompanied by a number of risk factors. The articular damage is an important feature and a challenge in this disease, since about 25-50 % of the patients have irreversible articular damage with total loss of joint function (11,12).

1.1.1 Risk factors

Septic arthritis can affect people at any age, but elderly people and very young children are more frequently affected (13,14). Furthermore, the presence of previous joint diseases, such as rheumatoid arthritis (RA), osteoarthritis, crystal arthropathies and other forms of inflammatory arthritis is a predisposing factor for the development of infectious arthritis. In particular, patients with rheumatoid arthritis (RA) have an approximately 10-fold higher incidence of septic arthritis than the general population (15,16). The incidence of septic arthritis does not increase only in previous arthritic patients, but is also more common in people that suffer from other chronic and

immunosuppressive diseases, such as diabetes, leukemia, cirrhosis, granulomatous diseases, cancer, hypogammaglobulinaemia, human immunodeficiency virus (HIV)-infected patients and intravenously drug users (17–19). Haemodialysis has been reported as an important risk factor for septic arthritis (20). Also, penetrating trauma, including animal bites and local therapeutic intra-articular corticosteroid injections may cause septic arthritis in atypical joints (21–23). Recent joint surgery is also associated with an increased risk for infection (24,25).

In addition, several cases of joint infections have been reported in patients that receive immunosuppressive therapy and/or glucocorticoids, which also constitute another important risk factor associated with the development of septic arthritis (26). In this context, the use of classic disease modifying anti-rheumatic drugs (DMARDs) in RA patients can be an additional risk factor that facilitates the development of infectious arthritis (27,28). Although data from observational registers have suggested an increased incidence of joint infections in patients receiving anti-tumor necrosis factor (TNF) therapy, the incidence does not seem to be different from the risk among patients treated with classical DMARDs (29).

1.1.2 Causative agents

The most common causative agent associated with septic arthritis is *Staphylococcus aureus*, which accounts for 52 % of cases (30). Recently, an increase in methicillin-resistant *S. aureus* (MRSA) infections has been reported in several health-care systems, particularly in the elderly and intravenous drug abuser populations as well as associated with orthopedic procedures (31). MRSA has been associated with 18 % of septic arthritis cases in a study in São Paulo, Brazil (32). Other bacteria such as group B streptococci, *Streptococcus pneumoniae*, *Neisseria gonorhoeae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus* genus and *Klebsiella* species can be associated with septic arthritis, but are less frequent (33). Common causative agents in children include *S. aureus*, *S. pneumonia* and *Kingella kingae* (34).

1.1.2.1 Staphylococcus aureus

Microorganisms of the genus *Staphylococcus* are gram-positive cocci with a common distribution in nature as part of the normal human microbiota (35). The bacteria can be classified into two groups according to their coagulase production: coagulase producers or *Staphylococcus aureus*, and coagulase negative *Staphylococci* (CNS) that include *S. epidermidis, S. haemolyticus, S. saprophyticus* and many others (36). *S. aureus* is the agent most often responsible for osteo-articular infection in practically all age groups and almost all forms of infection. The infective capacity of *S. aureus* in different tissues is provided by the presence of several virulence factors (37).

S. aureus presents a capsule that can be composed by polysaccharides and acts as a physical barrier that protects the bacteria from phagocytosis by immune cells (38). Peptidoglycan (PGN) is the major component of the cell wall of Gram-positive bacteria. PGN was detected in synovial tissue of patients with septic arthritis (39) and studies demonstrated that intra-articular injection of PGN in mice can cause arthritis (40). *S. aureus* is a bone pathogen because it possesses several cell-surface adhesion molecules that facilitate its binding to bone matrix (41). Binding involves a family of adhesins that interact with extracellular matrix components and these adhesions have been termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (42). Specific MSCRAMMs are needed for the colonization of specific tissues. Particular MSCRAMMs include fibronectin-binding proteins, fibrinogen-binding proteins, elastin binding and collagen-binding adhesion molecules. Once the bacteria adhere to and colonize bone matrix, they elaborate several virulence factors such as proteases, which can break down matrix components (43). Further experimental studies demonstrated that collagen adhesin is an important virulence determinant in *S. aureus*-induced arthritis (44).

S. aureus secretes a large number of enzymes and toxins, many of which have been implicated as potential virulence factors. Alpha and gamma toxins are lytic to red blood cells and various leukocytes, but not to neutrophils (45). The combination of these two toxins has been demonstrated experimentally to be important for the development of septic arthritis (46). Another toxin is Panton-Valentine leukocidin (PVL, consisting of the LukS and LukF proteins) that can lyse leukocytes, especially human neutrophils and is related with fulminant cases of septic arthritis (47). Enterotoxins, such as shock syndrome toxin-1 (TSST-1) can cause shock by stimulating

the release of interleukin (IL)-1, IL-2, TNF and other cytokines (48). Experimentally, the presence of TSST-1 favors the development of septic arthritis (49). Another important virulence factor is the bacterial deoxyribonucleic acid (DNA) with non-methylated CpG motifs, which is considerably less frequent in vertebrate DNA. (50). The CpG DNA can bind to Toll-like receptor 9 (TLR9) in immune cells and leads to the production of cytokines such as IL-1β, TNF, IL-6 and IL-12 (51,52). Some studies showed that intra-articular injection of *S. aureus* CpG DNA can induce arthritis (53,54).

1.1.3 Diagnosis

Gram staining and cultures of synovial fluid should be investigated in any case of undiagnosed arthritis. Antibiotic therapy ideally is advised until after synovial fluid is sampled (55). Gram stains of synovial fluid are helpful when positive, but they are not always sensitive enough for the diagnosis of septic arthritis (56). Patients should be treated empirically for septic arthritis when synovial fluid leukocyte counts exceed 50,000 cells/mm^3, although gout and pseudogout also commonly cause leukocyte counts of this magnitude (57). Thus, the analysis of the presence of urate crystals in synovial fluid by polarized light microscopy is very important for the exclusion of a gouty attack (58–60). However, the serum procalcitonin level appears to be a promising marker for septic arthritis (61). Blood cultures should be obtained in all patients with suspected septic arthritis, however the cultures must be obtained before starting antibiotic treatment to optimize the possibility of isolating the causative bacteria (62). DNA-based techniques, hybridization probes, polymerase chain reaction (PCR)-based techniques and detection of typical bacterial compounds by mass spectroscopy provide quick results (63). The detection of microorganisms by PCR has shown more accurate results (64). However, the risk of contamination, the presence of background DNA, the lack of a gold standard and the fact that PCR techniques detect DNA instead of living pathogens make the interpretation of these tests difficult (65).

Imaging can be used as complementary diagnosis since a computed tomography (CT) scan may not depict abnormalities during the early stages of infection. However, CT is a better imaging technique for visualisation of local oedema, bone erosions, osteitic foci and sclerosis (65). Magnetic resonance imaging (MRI) provides better resolution for the detection of joint effusion and for differentiation between bone and soft-tissue infections. MRI findings in patients with septic arthritis include joint

effusion, cartilage and bone destruction, soft-tissue abscesses, bone oedema and cortical interruption (66).

1.1.4 Treatment

Septic arthritis is so rapidly destructive that broad-spectrum antibiotics are usually warranted until culture data are available or bacteria have been identified by mass spectrometry. Given the increasing importance of MRSA as a cause of septic arthritis, initial antibiotic regimens should generally include an antibiotic active against MRSA, such as vancomycin (67). Cefazolin is a reasonable alternative in areas with a low prevalence of MRSA. If serious vancomycin allergy is present, empiric therapy utilizing linezolid or daptomycin must be considered (68) Septic arthritis associated with animal bites should be treated with agents active against oral microbiota, such as ampicillin-sulbactam (69).

In general, septic arthritis in adults should be treated for at least 3 weeks, which may include a period of step-down oral therapy (70). In children with uncomplicated septic arthritis, as few as 10 days of antibiotic therapy may suffice (71). Septic arthritis can be managed with antibiotics combined with joint drainage by arthroscopy, arthrocentesis, or arthrotomy. Joint drainage decompresses the joint, improves blood flow, and removes bacteria, toxins, and proteases (72). Arthrocentesis should be repeated daily until effusions resolve and cultures are negative. Aggressive rehabilitation is essential to prevent joint contractures and muscle atrophy (73).

1.2 Immune response against *S. aureus*

Pathogens are controlled by innate and adaptive immune responses and the recognition of microorganisms is the first step of host defense (74). In the joint, resident cells, such as synoviocytes, can recognize *S. aureus* through the pattern recognition receptors (PRRs). In that way, those cells produce inflammatory mediators such as cytokines, chemokines and lipids that will attract neutrophils and macrophages (75). In the outset of the inflammatory process, neutrophils are the main cell recruited to the site of infection and they have a fundamental role in both the phagocytosis and in killing the microorganism (76). The importance of neutrophils in controlling *S. aureus* in the joint was demonstrated in a study in which neutrophils were depleted. This caused the impairment of bacterial control (77). Other immune cells such as macrophages (78),

natural killer (NK) cells (78) and B lymphocytes (79) are described to have a role in experimental models of septic arthritis. Dendritic cells in *S. aureus*-induced arthritis are fundamental for the activation of the adaptive immune response. The depletion of dendritic cells during *S. aureus* infection in the lungs showed an increase in bacterial load and mortality (80). During *S. aureus* infection, dendritic cells can induce a Th1 response probably through IL-12 production. Experimentally, the lack of IL-12 increased the bacterial load in the joint systemically during *S. aureus*-induced septic arthritis (81). Dendritic cells can also stimulate Th17 activation, an important source of IL-17. The cytokine IL-17 has been shown to be important for bacterial clearance and to prevent tissue damage in experimental *S. aureus*-induced arthritis (82).

1.2.1 Neutrophils

Neutrophils are continuously generated in the bone marrow from myeloid precursors. Humans and mice differ in their numbers of circulating neutrophils. In humans, 50–70 % of circulating leukocytes are neutrophils, whereas this number drops to only 10–25 % in mice (83). In the circulation, mature neutrophils have a segmented nucleus and their cytoplasm is enriched with granules and secretory vesicles. After the first moments following infection, neutrophils can be recruited from blood vessels to the site of infection, a process that involves a close interaction between neutrophils and endothelial cells and is mediated by different chemotactic agents that activate the cells and guide their migration (84). Chemotactic factors for neutrophils include bacterial peptides (formylated methionyl-leucyl-phenylalanine) (85), products of complement activation (such as C5a) (86), extracellular matrix degradation products (laminin digests) (87), arachidonic acid metabolites (leukotriene B4) (88), other lipid mediators such as platelet activating factors (PAF) (89) and chemokines (89).

Neutrophils are recruited in a cascade of events that involves the following commonly recognized steps that precede the transmigration: tethering, rolling, adhesion, and crawling on the endothelial cell surface (90,91). Neutrophil recruitment is initiated by changes on endothelial cells during the early steps of inflammation. Endothelial cells can be activated directly by pathogens through PRR activation, causing an increase of the expression and exposure of adhesion molecules on their surface. Once on the endothelial surface, P-selectin and E-selectin bind to their glycosylated ligands on

leukocytes, leading to the tethering (capturing) of free-flowing neutrophils to the surface of the endothelium and subsequent rolling of neutrophils along the vessel in the direction of the blood flow (92). Rolling requires rapid formation and breakage of adhesive bonds. The rolling of neutrophils facilitates their contact with chemokine-decorated endothelium to induce activation. Full activation may be a two-step process initiated by specific priming by pro-inflammatory cytokines, such TNF and IL-1β, or by contact with activated endothelial cells followed by an exposure to pathogen-associated molecular patterns (PAMPs), chemoattractants or growth factors (93,94). The adhesion step of the recruitment cascade prepares neutrophils for transmigration, but migration does not necessarily occur at the initial site of their arrest on the endothelium. Some of the adherent neutrophils reveal so called crawling behavior as they elongate and continue to send out pseudopods, apparently actively scanning and probing the surroundings while remaining firmly attached to a single location within the microvasculature (95). During the transmigration process, neutrophils cross the endothelium in a process dependent on integrins. The passage across the endothelial cell layer occurs either paracellularly (between endothelial cells) or transcellularly (through an endothelial cell without mixing the cytoplasmic content of both cells). Next, neutrophils migrate towards the infectious/inflammatory focus in the tissue (96).

1.2.1.1 Neutrophil functions during infections

In order to kill microorganisms, neutrophils can phagocyte, secrete the content of their granules, produce reactive oxygen species (ROS) and antimicrobial peptides, and release neutrophil extracellular traps (NETs) (97). Once at the site of infection, the neutrophils bind and ingest invading microorganisms by phagocytosis, a critical first step in the removal of bacteria during infection. Neutrophils recognize numerous surface-bound and freely secreted bacterial products such as PGN, lipoproteins, lipopolysaccharide, CpG-containing DNA, and flagellin (98). Such conserved bacterial PAMPs (pathogen-associated molecular patterns) are recognized directly by PRRs expressed on the extracellular membrane or on organelles in the cytosol of the neutrophil (99). The process of neutrophil phagocytosis triggers synthesis of a number of immunomodulatory factors that will recruit additional neutrophils, modulates subsequent neutrophil responses, and coordinates early responses of other cells types

such as monocytes, macrophages, dendritic cells, and lymphocytes, thereby providing an important link between innate and acquired immune responses (100).

Phagocytosis is accompanied by the generation of microbicidal ROS (oxygen-dependent) and fusion of cytoplasmic granules with microbe-containing phagosomes (degranulation). Degranulation enriches the phagosome lumen with antimicrobial peptides and proteases (oxygen-independent process), which in combination with ROS create an environment non-conducive to survival of the ingested microbe (101). In the most classical sense, neutrophil activation is intimately linked with the production of superoxide and other secondarily derived ROS, an oxygen-dependent process known as the oxidative or respiratory burst. High levels of superoxide are generated upon full assembly of the multi-subunit nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase in both the plasma- and phagosomal membranes (102,103).

Neutrophils present three fundamental types of granules: primary or azurophilic, secondary or specific and tertiary or gelatinase-containing granules (104). Primary granules are the largest and are the first formed during neutrophil maturation. They are named for their ability to take up the basic dye azure A and contain myeloperoxidase (MPO), defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), and a number of serine proteases such as neutrophil elastase, proteinase 3 and cathepsin G (105). Granules of the second class are smaller, do not contain MPO and are characterized by the presence of the glycoprotein lactoferrin and antimicrobial compounds including neutrophil gelatinase-associated lipocalin, human cationic antimicrobial protein- 18 and lysozyme (106). The gelatinase granules, are also MPO-negative, are smaller than specific granules, and contain few antimicrobials, but they serve as a storage location for a number of metalloproteases, such as gelatinase and leukolysin (107). Neutrophils also present secretory vesicles that serve as a reservoir for a number of important membrane-bound molecules active during neutrophil migration. As a neutrophil proceeds through the activation process, granules are mobilized and fuse with either the plasma membrane or the phagosome, releasing their content into the respective environments (108).

Neutrophils produce peptides and proteins that directly or indirectly kill microbes. There are three main types of antimicrobials: cationic peptides and proteins that bind to microbial membranes, enzymes, and proteins that deprive microorganisms of essential nutrients (109). Many of these peptides disrupt the membrane integrity, whereas, some antimicrobials are thought to disrupt essential microbial functions, such

as DNA replication, transcription or production of energy (110). Recently, it was demonstrated that neutrophils can produce neutrophil extracellular traps (NETs) which contain decondensed chromatin, bound histones, azurophilic granule proteins and cytosolic proteins. They have a demonstrated capacity to bind to and kill a variety of pathogens including *S. aureus* (111). Extrusion of such structures by neutrophils is predicted to limit microbial spread and dissemination, while enhancing effective local concentrations of extruded microbicidal agents, thereby promoting synergistic killing of attached microorganisms (112).

All the mechanisms used by the neutrophil to eliminate the pathogen can also cause host tissue damage (113). In that way, neutrophil recruitment needs to be tightly controlled and neutrophils must be removed before they have serious, detrimental effects on inflamed tissues. Once neutrophils have executed their antimicrobial function, they die via a built-in cell-death program. However, not only does apoptosis reduce the number of neutrophils present, it also produces signals that abrogate further neutrophil recruitment (114).

1.2.1.2 The chemokine system in neutrophil recruitment

Chemokines are small proteins with molecular mass of ~7-12 kDa that belong to the family of chemotactic cytokines. Chemokines are the only group of cytokines that bind to G protein-coupled receptors (GPCRs) (115). Chemokines were named based on their chemoattractant property, described first in 1987 when CXCL8 was shown to be involved in chemotaxis of neutrophils *in vitro* (116). Additionally, chemokines were described to be involved in other functions such as embryogenesis, homeostasis, angiogenesis and inflammation (117). Chemokines can be divided into 4 subfamilies based on the position of the two cysteine residues in their N terminal amino acid sequence: 1) CC chemokines have two adjacent cysteines; 2) CXC chemokines present one amino acid between the two cysteines; 3) the CX3C chemokine has 3 amino acids between the cysteines, and; 4) C chemokines lack one of the two N-terminal cysteines (118). The ELR^+ CXC chemokines that have a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (ELR) immediately before the first cysteine of the CXC motif are are associated with neutrophil recruitment and include CXCL1, 2, 3, 5, 6, 7 and 8. Those without an ELR motif rather recruit T and B lymphocytes or haematopoietic precursor cells (119,120).

Chemokines can bind to two types of receptors: GPCRs and atypical chemokine receptors (ACKRs) that do not signal through G proteins and lack chemotactic activity. GPCRs are classified as CCR, CXCR, CX3CR and XCR according to the cysteine motif in their ligands (121). The interactions of chemokines with those receptors are shown in Table 1. As can be seen, one chemokine can bind to several receptors and one receptor may transduce signals for different ligands. These interactions, that at the first moment were considered as "redundant" gave rise to the term promiscuity of the chemokine system. However, much attention is given now to the "bias of the chemokine system", including ligand bias, receptor bias and tissue bias, which tend to explain and allow us to understand how those chemokines bind to their receptors and promote different responses in different situations (122).

GPCRs have seven transmembrane helices with three extra and three intra cellular loops, an extracellular N terminus and intracellular C terminus. Chemokines bind to the extracellular domain and to a pocket in the transmembrane area and the signal is transmitted to the intracellular compartment. Cells are activated by the direct coupling of the G proteins or β arrestins (123). The intracellular signaling in the chemokine receptors is related to second messengers such as calcium, cyclic adenosine monophosphate (cAMP) and GTPases (Ras and Rac). The GPCRs can also signal through β arrestins, a pathway that can regulate the receptor signal through the desensitization process (124). β arrestins can block the binding to the phosphorylated G proteins and they are responsible for internalization of receptors to endosomes and degradation. Desensitization may be critical for maintaining the capacity of the cell to sense a chemoattractant gradient (125). Multiple ACKRs have been reported to signal through β arrestins (122).

Table 1: Chemokine and chemokine receptor family

TABLE 1		
CHEMOKINE RECEPTORS	**HUMAN CHEMOKINE LIGANDS**	**MURINE CHEMOKINE LIGANDS**
CC receptors		
CCR1	CCL3, CCL3L1, CCL4L1, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, CCL23	CCL3, CCL5, CCL6, CCL7, CCL9
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16	CCL2, CCL7, CCL12

continued		
CCR3	CCL3L1, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28	CCL5, CCL7, CCL9, CCL11, CCL14, CCL24, CCL26, CCL28
CCR4	CCL17, CCL22	CCL17, CCL22
CCR5	CCL3, CCL3L1 CCL4, CCL4L1, CCL5, CCL8, CCL11, CCL14, CCL16	CCL3, CCL4, CCL5
CCR6	CCL20	CCL20
CCR7	CCL19, CCL21	CCL19, CCL21
CCR8	CCL1, CCL16, CCL18	CCL1, CCL8
CCR9	CCL25	CCL25
CCR10	CCL27, CCL28	CCL27, CCL28
CXC receptors		
CXCR1	CXCL6, CXCL8	CXCL1, CXCL2, CXCL6, CXCL7, CXCL8
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	CXCL1, CXCL2, CXCL3, CXCL6, CXCL7
CXCR3	CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11	CXCL4, CXCL9, CXCL10, CXCL11
CXCR4	CXCL12	CXCL12,
CXCR5	CXCL13	CXCL13
CXCR6	CXCL16	CXCL16
CXCR8	CXCL17	CXCL17
XC receptor		
XCR1	XCL1, XCL2	XCL1, XCL2
CX3C receptor		
CXR1	CX3CL1	CX3CL1
Atypical receptors		
ACKR1	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL4, CXCL4L1, CXCL5, CXCL6, CXCL8, CXCL11	CCL2, CCL5, CCL7, CCL11, CCL17, CXCL4, CXCL6, CXCL11
ACKR2	CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22	CCL2, CCL3, CCL4, CCL5, CCL8, CCL11, CCL17, CCL22
ACKR3	CXCL11, CXCL12	CXCL11, CXCL12
ACKR4	CCL19, CCL21, CCL25	CCL19, CCL21, CCL25
UNKNOWN	CXCL14	CXCL14, CXCL15, CCL18

In total, 20 chemokine receptors are described and they are all expressed in leukocytic cells. Based on their functions, they can be divided into constitutive and inducible or homeostatic and inflammatory receptors. Initially, inflammatory chemokines and their receptors were only studied in the context of inflammation, but some receptors were identified as co-receptors for HIV entrance into the cell and others are associated with tumor metastasis (126,127). Regarding homeostasis, the chemokine system is involved in embryogenesis, leukocyte trafficking to lymphoid organs, tissue/organ development and angiogenesis. For instance, much attention has been given to the contribution of the CXCR4 receptor in embryogenesis, hematopoiesis, and leukocyte traffic from bone marrow. The importance of CXCR4 in this condition is critical for survival, since the deletion of CXCR4 or its ligand CXCL12 in mice is embryonically lethal (128,129). Chemokines are also important for T and B cell development. The development of T lymphocytes in the thymus is dependent on CCL21, CCL25, CXCL12, CCR7, CCR9 and CXCR4 (130). In bone marrow the CXCL12/CXCR4 axis is related to the development of B cells, monocytes, macrophages, neutrophils, natural killer (NK) cells, and plasmacytoid dendritic cells (131). Chemokines have a role in angiogenesis in health and disease. The ELR^+ CXC chemokines, all ligands for CXCR2, have angiogenic activity. In contrast, the ELR^- CXC chemokines such as CXCL4, CXCL4L1, CXCL9-11, all ligands for CXCR3, are angiostatic. Furthermore, the chemokines CCL1, CCL2, CCL5, CCL11, CCL15 and CCL16 also significantly contribute to angiogenesis (132). During inflammation, the fundamental function of chemokines is cellular recruitment to the inflamed tissue: CXCR3 and CXCR4 are important for T lymphocyte recruitment; CXCR5 for B cell recruitment; CCRs for monocyte, lymphocyte, NK cell, eosinophil, and basophil recruitment (133). CXCR1 and CXCR2 were the first members of the chemokine receptor family to be cloned, sharing a high degree of homology with formyl peptide receptors (FPRs) (134). Neutrophils express high levels of CXCR1 and CXCR2 on their surface once activated and the receptors and their ligands have an important role in neutrophil recruitment (135).

Another group of chemokine receptors, the ACKRs also have seven transmembrane domains, but they are not able to activate G proteins. Four members of this group had been described until now, ACKR1-4 (136). Those receptors are expressed in leukocytes and in non-hematopoietic cells. ACKRs signal through the β

arrestin pathway, but they also work as scavenger receptors, since they can internalize the bound chemokines without chemotactic actions (137).

1.2.1.2.1 Regulation of the chemokine system

Chemokine activity can be regulated at multiple levels, including gene duplication, gene transcription and translation. Some pre-formed chemokines are stored in endothelial cells, inside secretory granules including Weibel–Palade bodies, and are quickly released upon cell insult (138). Once produced, the chemokine activity can be regulated by binding to glycosaminoglycans on endothelial cell layers of lymph and blood vessels, by the expression and binding to GPCRs and ACKRs, or by receptor-mediated synergy and antagonism among chemokines (139). Recently, microRNAs, regulating the chemokine and chemokine receptor mRNA levels, were discovered as a novel mechanism for fine-tuning chemokine and chemokine receptor expression (140). Finally, chemokines and their receptors become post-translationally modified. Chemokines can be modified post-translationally through: (1) proteolytic cleavage by enzymes such as metalloproteinases, CD26 and enzymes from pathogens (141); (2) citrullination, that is the formation of citrulline by the deimination of arginine by peptidylarginine deiminases (PAD) (142); (3) N-glycosylation on asparagine within an Asn-Xaa-Ser/Thr motif, or O-glycosylation on serine (Ser) or threonine (Thr) residues (143); and (4) nitration, where peroxynitrite produced during oxidative stress can selectively oxidize and nitrate several residues, including the oxidation of histidine and the nitration of tyrosine and tryptophan (144). Reduced or enhanced receptor affinity and chemokine activity have been reported, depending on the chemokine and on the type of posttranslational modification (141). Most posttranslational modifications on inflammatory chemokines are depended on proteolytic cleavage, mainly affecting the NH_2-terminal region of the protein with highly specific proteases (126,127).

1.2.1.2.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear carbohydrate structures, consisting of a repeating disaccharide unit, that comprises a hexuronic acid linked to an N-acetyl-hexosamine that can be sulfated at different positions. GAGs are negatively charged and have a molecular weight around 10-100 kDa (147). GAGs are divided in 6 groups: heparan sulphate, heparin, chondroitin sulphate, dermatan sulphate, keratan sulphate

and hyaluronic acid. These sugar units can bind or attach to protein cores of proteoglycans or can be found associated with the extracellular matrix. GAGs are heterogeneous in length and composition and they can bind to a huge number of proteins (148). GAGs have fundamental roles in cell signaling and development, angiogenesis, tumor progression, embryogenesis, wound healing, and have anti-coagulant properties (133). Interestingly, GAGs can interact directly with pathogens. Particularly related to this study, hyaluronic acid favors to increase lubrification in synovial joints. The loss of hyaluronic acid in osteoarthritic patients is associated with an increase of pain and stiffness (122).

Each tissue produces specific GAG repertoires and cells can alter their GAG expression in response to specific stimuli or in pathologic states. GAGs are important in cell recruitment during homeostatic and inflammatory processes by their direct interaction with chemokines (149). The binding of chemokines to GAGs can generate an immobilized chemokine gradient that directs cell migration, as shown in Figure 1. Cell surface immobilization of chemokines enables them to act locally rather than as paracrine molecules, and likely prevents inappropriate activation and desensitization of receptors on cells outside the region of interest for a given physiological situation (150).

Almost all chemokines are basic proteins, often with a pI of 10 or higher, with many Arg, Lys and His residues and GAGs bind to proteins with positive charges. The epitopes for GAG binding on chemokines are described to be BBXB and BBBXXB motifs, where B represents a basic amino acid (151). It has been shown that some chemokines act as monomers, whereas many chemokines can oligomerize and form diverse quaternary structures including dimers, tetramers and polymers, increasing the number of epitopes that bind to GAGs (148). Oligomerization increases the affinity of chemokines for GAGs through an avidity effect and this interaction also stabilizes the chemokine oligomers. Moreover, oligomerization may have a dramatic effect on GAG affinity and specificity (152).

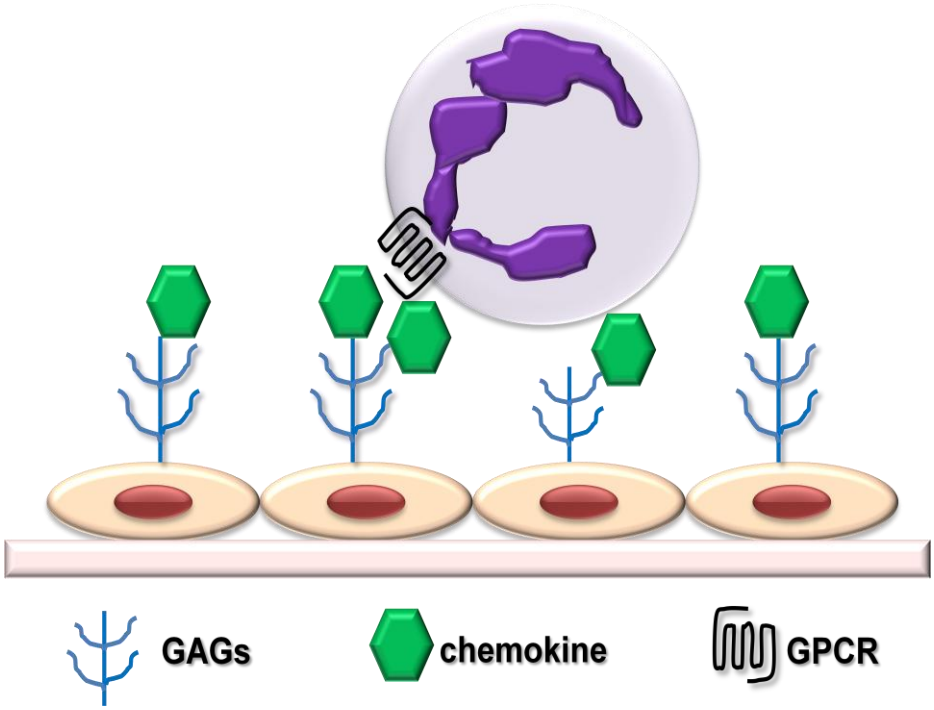

Figure 1: Role of the interaction between chemokines and glycosaminoglycans

1.2.1.2.3 Chemokines as therapeutic targets

The knowledge about the chemokine system in the context of inflammatory diseases has been characterized in more detail in recent years. That has encouraged the development of different compounds that target chemokines and their receptors to be investigated for therapies. For instance, the blockade of chemokine receptors could be performed by neutralizing antibodies, as demonstrated by the treatment with anti-CXCR3 antibody during heart allograft transplantation in mice. This treatment produces the same protective effect as deletion of the CXCR3 gene in an attempt to avoid rejection (153). One could also use posttranslationally modified chemokines. It was demonstrated that the truncated form of CCL2 works as an antagonist of CCR2 and protected tissue damage in a model of spontaneous arthritis (153). Furthermore, the synthesis of new molecules that antagonize chemokine receptors has been developed elsewhere (154). In this context, the small molecule receptor antagonist DF2156A acts as an allosteric non-competitive antagonist of CXCR1/2 and has been investigated for its potent anti-arthritic effect (122). Controlling the activity of chemokines by the development of modified chemokines also is a promising strategy to impair the function of chemokines during inflammatory responses. For example, our group has used

different isoforms of chemokines and chemokine-derived peptides that compete with intact chemokines for GAG binding(155,156). For instance, the secreted ELR^- CXC chemokine CXCL9 consists of 103 amino acids and attracts activated T lymphocytes and NK cells through activation of the CXCR3 (157). In previous publications, we showed that a COOH-terminal fragment of CXCL9 [CXCL9 (74-103)] is able to compete with CXCL8 for GAG binding and reduces neutrophil recruitment towards intraperitoneally or intra-articularly injected CXCL8 and in the cremaster muscle and monosodium urate crystal-induced gout models (158,159).

1.2.1.3 The 5-Lipoxygenase pathway: mechanisms of neutrophil recruitment and inflammation

At the onset of inflammation, classic lipid mediators are produced, including leukotriene B_4, which activate and amplify the cardinal signs of inflammation (160). 5-lipoxygenase (5-LO) is the main enzyme involved in the production of these lipid mediators. This enzyme is expressed in leukocytes such as neutrophils, macrophages, dendritic cells, B cells and T cells (161). During the inflammatory process, another class of arachidonic acid (AA) derived lipids, prostaglandins E_2 and D_2, induce the switch of leukotriene synthesis to pro-resolving lipid production, including lipoxin A_4 (LXA_4) (162,163). The synthesis of LXA_4 is also dependent on 5-LO. LXA_4 has an important role in the resolution of inflammation by decreasing neutrophil migration and on the other hand increases recruitment of macrophages. Additionally, LXA_4 increases the phagocytosis of apoptotic neutrophils by macrophages, a process named efferocytosis, to avoid tissue damage (164).

1.2.1.3.1 Leukotriene B4

Leukotriene B_4 (LTB_4) is a very potent chemoattractant for neutrophils. LTB4 is produced from AA in a pathway dependent on lipoxygenases (LO) (88) as shown in Figure 2. AA is a 20-carbon fatty acid and the main eicosanoid precursor and is present in all cells. Some stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), CXCL8, microorganisms, phagocytic particles and damage or injury can activate phospholipases and release AA from the cell membrane (165). In the cytosol AA can be metabolized in leukotrienes and lipoxins in a pathway dependent on LO. The main LO

enzymes are 5-LO that is expressed in leukocytes and 12/15-LO expressed in reticulocytes, eosinophils, immature dendritic cells (DCs), epithelial and airway cells, pancreatic islets and resident peritoneal macrophages (166). The first step in leukotriene biosynthesis is the conversion of AA into a hydroperoxide, named 5-hydroperoxyeicosatetraenoic acid (5-HPETE), by the insertion of an oxygen at position 5. In this step the activation of 5-LO is dependent on the 5-LO activating protein (FLAP). 5-HPETE can be reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) or can be converted in a 5,6-epoxide containing a conjugated triene structure, named leukotriene A_4 (LTA_4) by removal of a water molecule (167). LTA_4 is instable and can be converted to leukotriene B_4 (LTB_4) by insertion of a hydroxyl group at carbon 12 by the enzyme LTA_4 hydrolase. Another possibility is the conversion in leukotriene C_4 (LTC_4) by addition of a glutathionyl group at carbon 6 by γ- glutamyl-S-transferase (168). LTB_4 is produced and released within minutes by neutrophils, macrophages, and mast cells and is an important element of the immediate inflammatory response(169).

Leukotrienes bind to extracellular GPCRs which are members of the rhodopsin-like receptors family and related to chemokine receptors. LTB_4 is described to bind to 2 LTB4 receptors named BLT1 and BLT2 (170). BLT1 is a 43 kDa GPCR expressed in inflammatory cells such as neutrophils, alveolar macrophages, eosinophils, differentiated T cells, dendritic cells and osteoclasts and has a high affinity for LTB_4. The BLT2 receptor has low affinity for LTB_4 and is expressed more ubiquitously (171). BLT1 is widely related to chemotaxis. The axis LTB_4/BLT1 is needed for neutrophil recruitment in arthritis and for the recruitment of neutrophils to lymph nodes during bacterial infection. On the other hand, the axis LTB_4/BLT2 is involved in the generation of reactive oxygen species and can enhance would healing (169,172,173).

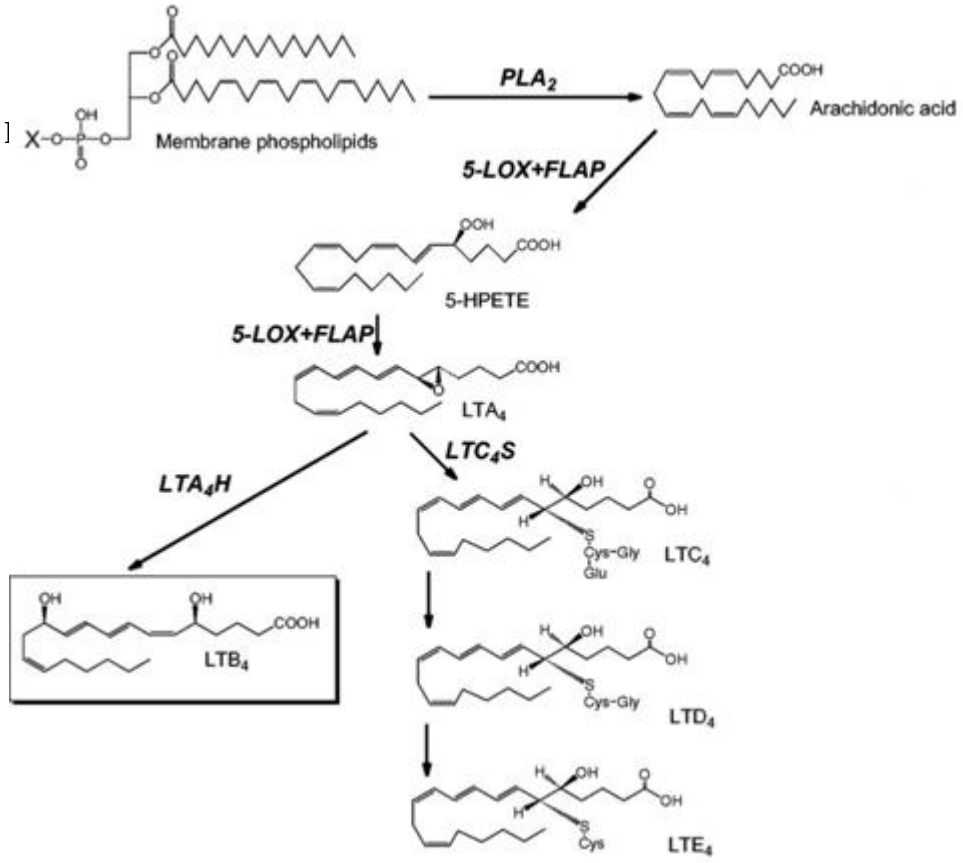

Figure 2: Production of leukotrienes from membrane phospholipids (171)

1.2.1.3.2 Lipoxin A_4

Lipoxins can be generated by three main pathways, as demonstrated in Figure 3. In the first one, AA is released from the cell membrane and one oxygen is inserted at carbon 15 by 15-LO in eosinophils, monocytes or epithelial cells, resulting in the intermediate 15S-HPETE. 15S-HPETE can be taken up by neutrophils or monocytes and converted in the 5,6-epoxytetraene by 5-LO and then is hydrolyzed by lipoxin A_4 or lipoxin B_4 hydrolases in lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) (174,175). The second route involves the interaction between leukocytes and platelets. The 5-LO present in leukocytes, such as neutrophils, converts AA into LTA_4 as described before. The LTA_4 is released and taken up by adherent platelets. These express 12-LO that converts LTA_4 in LXA_4 and LXB_4 (176). The third route occurs after the exogenous administration of aspirin. In this case, aspirin triggers the formation of the 15R-epimer of lipoxins, 15-epi-LXA_4 and 15-epi-LXB_4. These epimers carry a carbon 15 alcohol

group in the R configuration. They arise from aspirin-acetylated by cyclooxygenase-2 (COX-2) and share the actions of LXA_4 (177).

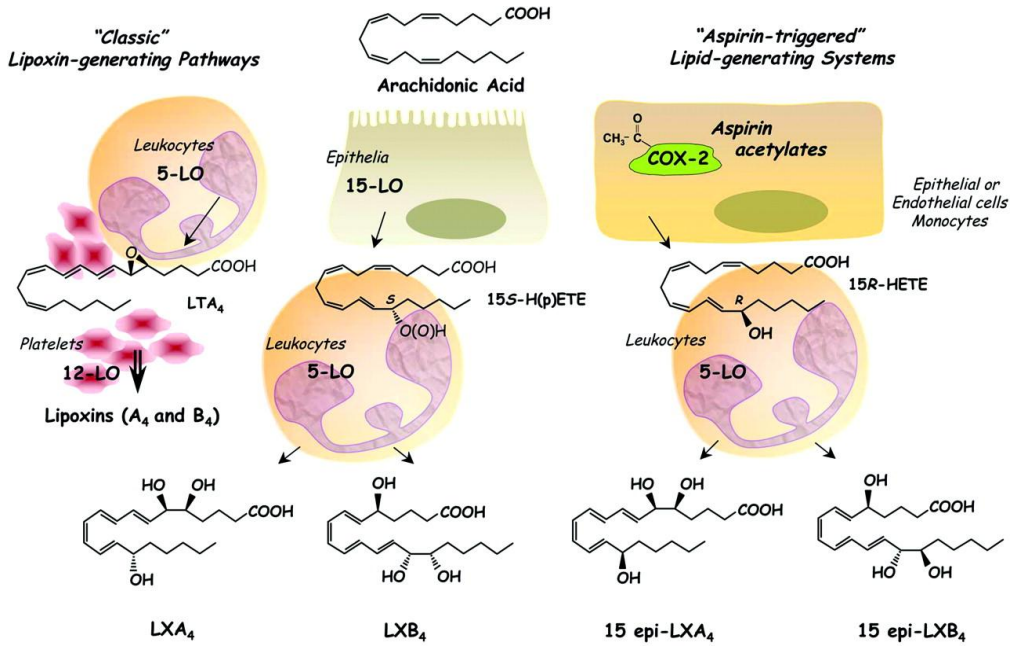

Figure 3: Three main pathways in the synthesis of lipoxins (305)

LXA_4 binds to the GPCR receptor ALX/FPR2. ALX is expressed in leukocytes, astrocytoma cells, epithelial cells, hepatocytes, microvascular endothelial cells and neuroblastoma cells. Unlike classic GPCRs for chemoattractants that mobilize intracellular Ca^{2+} to evoke chemotaxis, lipoxins instead induce changes in the phosphorylation of proteins of the cytoskeleton, resulting in β arrestin activation (178,179). LXA_4 presents pro-resolving actions such as decreased neutrophil infiltration, increased recruitment of mononuclear cells and an increase of the uptake of apoptotic neutrophils by macrophages. LXA_4 has also effects on the return of vascular permeability to the normal levels (180).

Both lipids LTB_4 and LXA_4 have been described to be involved in articular diseases. LTB_4 and 5-LO mRNA was found in synovial tissue of patients with rheumatoid arthritis (181,182). LTB_4 is also associated with pathogenesis in the collagen-induced arthritis model, the K/BxN serum transfer arthritis model(183–185) and the experimental model of gout (186). LXA_4 was also detected in synovial tissue of

patients with rheumatoid arthritis (187). Nonetheless, in zymozan-induced arthritis LXA_4 was related to attenuation of the disease(188). During infection LTB_4 and LXA_4 are related to clearance of pathogens and improvement of the disease. Some studies show that LTB_4 has a role in the control of lung Paracoccidioidomycosis (189) and is important for phagocytosis and killing of *Borrelia burgdorferi* (190). In lung infection by *Cryptococcus neoformans* (191) and sepsis (192), LXA_4 is associated with the control of infection and an increase in survival. However, in pneumosepsis induced by *Klebsiella pneumoniae*, the LXA_4 in the early stage of the disease is associated with systemic infection-induced mortality and at a late stage of the disease can improve survival (193).

Based on the aforementioned mechanisms on the pathology of septic arthritis, this disease is associated with severe articular damage and pain. During the immune response against *S. aureus* infection, neutrophils are the main cells recruited to the joint. Neutrophils are important in the control of infection. However these cells are also associated with articular damage and pain development (194,195). In sterile inflammation, the pharmacological blockade of neutrophil migration to the tissue is a very promising strategy to avoid or decrease tissue damage and dysfunction (196,197). However, the benefit of the blockade of neutrophil recruitment in infectious diseases is less clear. Thus, we initially hypothesized that the blockade of neutrophil migration and activity could be an interesting strategy to avoid excessive articular damage and pain during *S. aureus*-induced septic arthritis in mice. We focused our attention on the neutrophil-related chemoattractants, such as chemokines and 5-LO metabolic product, LTB_4. Regarding the chemokine biology, we used an antagonist of CXCR1/2 and a chemokine fragment that competes with active chemokines for GAG binding. With respect to the 5-LO pathway, the experiments on 5-$LO^{-/-}$ mice revealed a very important function of LXA_4 on septic arthritis development. We also investigated the role of this pro-resolving lipid on the development of septic arthritis.

2. GENERAL AIM

Neutrophils are important innate immune cells for the clearance of bacteria from infected tissue. However, neutrophil infiltration in tissues also causes a number of side effects. S. aureus injection is the most common cause of septic arthritis. In septic arthritis, bacterial clearance by the immune system is associated with severe joint damage. In this PhD thesis we wish to evaluate in detail the role of neutrophils and the enzyme 5-lipoxygenase in joint inflammation, damage and dysfunction in *S. aureus*-induced arthritis in mice.

2.1 specific aims

1. To evaluate joint inflammation, tissue damage and hypernociception after the injection of *S. aureus*
2. To investigate the effects of blockade of two main chemokine receptors involved in the migration of neutrophils, i.e. CXCR1 and CXCR2, on the pathogenesis of *S. aureus*-induced arthritis
3. To investigate the effects of treatment with the glycosaminoglycan-binding chemokine-derived peptide CXCL9(74-103) on the pathogenesis of *S. aureus*-induced arthritis
4. To investigate the role of the enzyme 5-lipoxygenase, focusing on the molecule LXA_4, on the pathogenesis of *S. aureus*-induced arthritis

3. MATERIALS AND METHODS

3.1 Mice

All animals experiments were performed according to the rules of the Ethics Committee of UFMG (protocol nº. 236 / 2012). Part of the experiments was performed in Belgium according to the protocol P 141/2015 approved by Ethics Committee for animal experiments of KU Leuven. Eight-to-ten-week-old male mice were used for all strains. C57BL/6J mice were purchased from the Centro de Bioterismo of Universidade Federal de Minas Gerais and SV 129, 5-LO$^{-/-}$, CD11c DTR mice [this animal possesses the diphtheria toxin receptor (DTR) driven by the CD11c promoter] were maintained in the animal facility of UFMG. Intraperitoneal injection of diphtheria toxin (4ng/g) causes the depletion of CD11c expressing cells in DTR mice. CD11c YFP mice (these animals have CD11c cells stained with yellow fluorescent protein YFP) were also maintained at UFMG. In Belgium, C57BL/6J mice were purchased from Janvier Labs. The animals were maintained under suitable conditions with free access to water and food and a light-dark cycle of 12 hours.

3.2 Bacteria

The *S. aureus* American Type Culture Collection (ATCC) 6538 strain was kindly provided by Professor Waldiceu Verri Jr from Universidade Estadual de Londrina. The inoculum was prepared by growth in brain heart infusion agar (BHI) supplemented with 5 % sheep blood for 24 hours at 37°C. The bacterial solution was prepared in phosphate buffered saline (PBS) at a concentration of 10^7 colony-forming units (CFU)/mL

3.3 Chemokines and Drugs

CXCL8 and CCL21 were purchased from R&D Systems. For *in vitro* experiments CXCL8 was used at concentrations 1.25 µg/ml, 2.5 µg/ml or 5 µg/mL and CCL21 at 10 ng/mL, 30 ng/mL or 100 ng/mL). The non-competitive allosteric inhibitor

DF2156A was kindly provided by Dompé Pharma- Italy, diluted in carboxymethylcellulose (CMC) 5% and used at a concentration of 10 mg/kg by gavage. The antibiotic vancomycin (Teuto) was diluted in PBS and used at 400 mg/kg by intraperitoneal injection. BOC2 (an FPR1/2 antagonist - Phoenix Pharmaceuticals) and WRW4 (an FPR2 antagonist - Tocris bioscience) were diluted in PBS and were given at 2 mg/kg (intraperitoneally – i.p.) or 10 µg/10µL (intra-articularly – i.a.), respectively. LXA_4 (Cayman Chemical), was diluted in PBS and used at 20 ng/10µL by intra-articular injection for *in vivo* experiments and at 100 ng/ml, 300 ng/ml or 1000 ng/mL for *in vitro* studies. The experimental approach for each drug is described in detail in the Results section and in the Figures legends.

3.4 Experimental model of septic arthritis

The inoculum of *S. aureus* was prepared as described above. Mice were placed under anesthesia by intraperitoneal injection of 60:5 mg/kg ketamine:xylazine. The hair over the knee was removed (Figure 4A, B) to find the articular cavity (Figure 4C). Ten microliters containing 10^7 CFU/mL of the bacteria were injected into the tibiofemoral knee joint of mice (Figure 4D). Viable counts were used to check the concentration of injected bacteria.

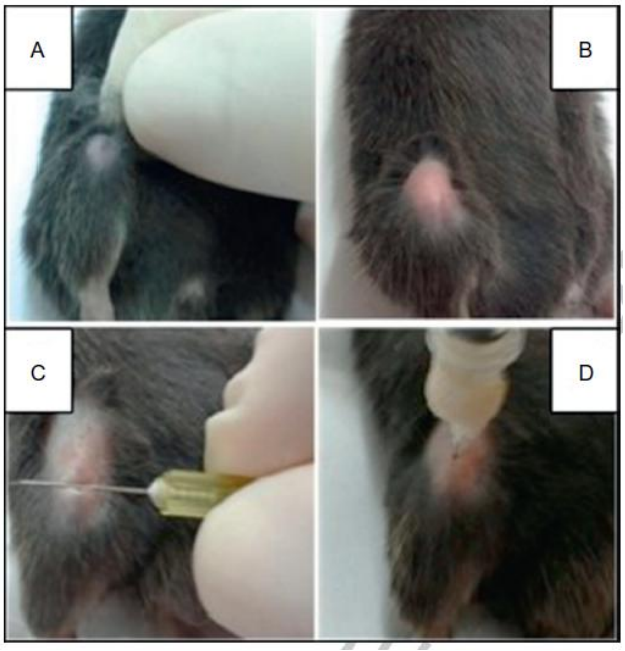

Figure 4: Procedure for intra-articular injection. The mouse hair covering the knee is removed (A and B), the articular cavity is found using a needle (C) and the articular injection is performed using an insulin syringe (198).

3.5 Intra-articular lavage and cell counting

Groups of mice were anesthetized as described before, culled for cervical dislocation and the skin over the knee was removed as demonstrated in Figure 5A. Then, the tendon was cut (Figure 5B), the articular cavity was exposed (Figure 5C) and the lavage (Figure 5D) was performed by washing the cavity twice with 5 µL of PBS containing 3 % bovine serum albumin (BSA). The periarticular tissue demonstrated in Figure 5C by the red dashed line was removed and processed to measure chemokine and cytokine levels. The number of leukocytes from the articular cavity was determined in a Neubauer chamber, after staining the cells with Turk's solution (RenyLab Chemistry). Differential counts were performed on Cytospin (Shandon III) preparations by evaluating the percentage of each leukocyte on a slide stained with May-Grunwald-Giemsa (Interlab).

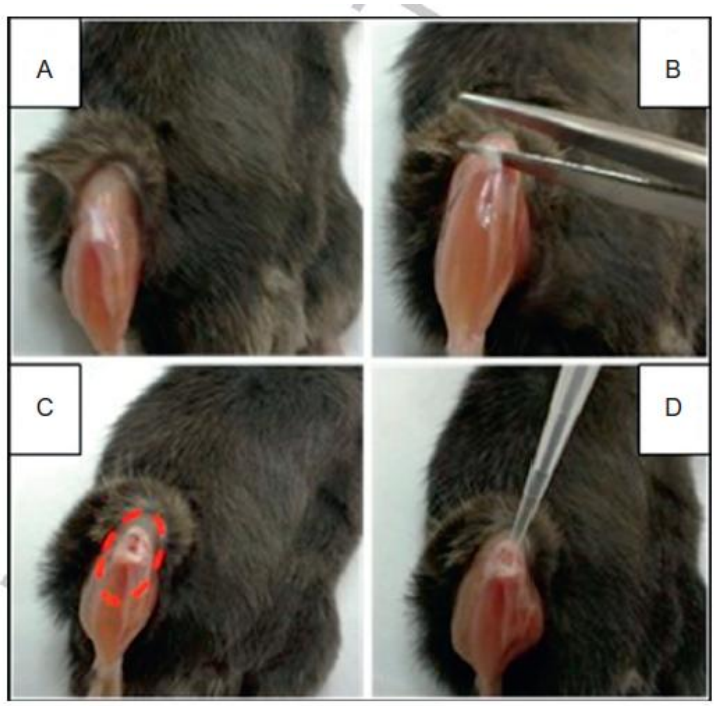

Figure 5: Procedure for articular lavage and collection of periarticular tissue. The skin under the knee is removed (A), the tendon is cut (B), and the articular cavity is exposed (C). The dashed line represents the periarticular tissue that was removed for enzymatic assays (C). The procedure demonstrating the recovery of cells from the cavity is shown in panel D. (198)

3.6 Procedure for the quantification of the bacterial load

The bacterial load in the joint was assessed by the complete removal of the knee tissue. The tissue was macerated in sterile PBS solution using a mortar and pestle. A serial dilution of the sample was performed in PBS and 10µL of each dilution were plated in brain heart infusion (BHI) agar supplemented with blood for 24 hours at 37ºC to evaluate the bacterial load. The bacterial colonies were counted and expressed in colony-forming units (CFU) /tissue.

3.7 Measurement of chemokines, cytokines, LTB_4 and LXA_4 by enzyme-linked immunosorbent *assay (ELISA)*

Periarticular tissue was collected and homogenized in a cytokine extraction solution [0.4 M NaCl, 10 mM $NaPO_4$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM

Benzethonium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.05% Tween 20, 0.5% BSA, 20 IU de aprotinin] using a tissue homogenizer (Power Gen 125 - Fisher Scientific Pennsylvania, EUA). Samples were centrifuged for 10 minutes at 10.000 rpm and the supernatant was used to evaluate cytokine and chemokine concentrations, in accordance with the manufacturer's instructions (R&D Systems). The capture antibody of interest was added to a 96-well plate and incubated for 24 hours at 4 °C. Subsequently, the plates were washed 3 times with PBS containing 0.05% Tween 20 and blocked for 2 hours with PBS containing 1% BSA at room temperature. Standards and 3-fold diluted samples (in 1x PBS containing 0.1 % BSA) were incubated for 24 hours at 4 °C. The next day the plates were washed 3 times (with PBS containing 0.05 % Tween-20) and incubated in the presence of detection antibodies. After 2 hours, a solution containing streptavidin bound to peroxidase was added to the plate. After 20 minutes, the plates were washed again and buffer containing orthophenylenediamine (OPD) was added. The reaction was stopped by the addition of H_2SO_4. The optical density was quantified using the ELISA plate reader at a wavelength of 490 nm. The result was expressed in pg/mL or ng/mL.

LXA_4 and LTB_4 were extracted from the joint tissue using a C18 Sep-Pak column and quantified using a specific ELISA as described by the manufacturer (Cayman Chemical). The samples and a biotin-conjugated target antigen were added to a 96 well plate pre-coated with a specific capture antibody and incubated for 1 hour. The plate was washed 3 times and subsequently Avidin-Horseradish Peroxidase (HRP) conjugate was added which bound to the biotin. Unbound HRP-conjugate was washed away and tetramethylbenzidine (TMB) substrate was added which reacted with the HRP enzyme resulting in color development. The reaction was stopped by the addition of H_2SO_4, and the optical density (OD) of the well was measured at a wavelength of 450 nm.

3.8 Evaluation of Hypernociception

Hypernociception was evaluated by the paw pressure test, also known as von Frey electronic method (199). Mice were packed in acrylic boxes that are placed on a shelf. This shelf provides as floor with a mesh net that allows the use of a mirror attached 25 cm below the boxes with inclination of approximately 35 degrees allowing the visualization of the paws of the animals (Figure 6 A, B). A digital pressure gauge

consisting of a pressure transducer connected to a digital force counter expressed in grams (g) with an accuracy of 0.1 g was used for the test. For the contact of the pressure transducer with the animal paw, a disposable 0.5 mm polypropylene tip was adapted (Figure 6A). Thus, a manually and linearly increasing pressure was performed in the plantar region of the paw of the animals, so as to produce a characteristic response of stimulated paw withdrawal. Results were expressed as the change in withdrawal threshold (in grams).

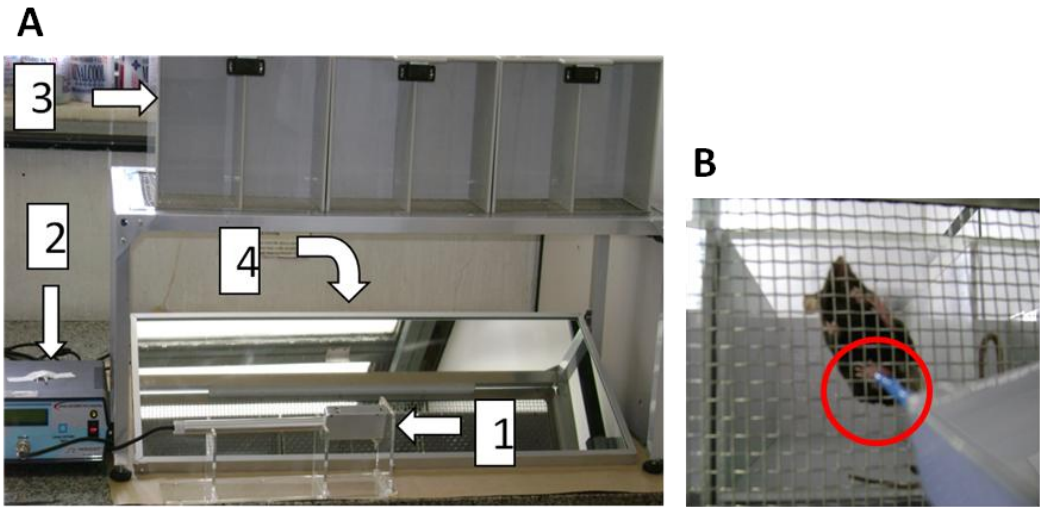

Figure 6: Procedure for hypernociception measurement. (A) The pressure sensor (1) is connected to an apparatus (2) which reports, in grams, the load supported by the animals. Mice are packed into the acrylic boxes (3) and, through the visualization of the paw in the mirror (4), are stimulated until a characteristic paw withdrawal response is observed. (B) The stimulation with the analgesimeter tip occurs through the grid.

3.9 Histopathological analysis

The articular tissue was collected and conditioned in histological cassettes that remained for 48 hours in 10 % buffered formalin solution. The cassettes were then transferred to an EDTA solution (14%) for bone decalcification for 30 days. During this period the EDTA solution was changed every 48 hours. The tissue processing was performed by subsequent passages in ethanol at different concentrations (80%, 90% and absolute ethanol; 1 and 2 - 30 minutes each), xylol (1 and 2 - 20 to 30 minutes each) and included in paraffin. Microtome sections were cut and one section was stained with

Harris Hematoxylin (20 seconds) and Eosin (8 seconds). The sections were analyzed under light microscopy and the following parameters were observed: severity of synovial hyperplasia (0-3), intensity and extension of the infiltrate (0-4) and bone resorption (0-2).

A second section was used to evaluate the content of proteoglycan. Initially the paraffin was removed from the section and hydrated in xylene (1, 2 and 3) and alcohol (absolute ethanol followed by 70% and 50% ethanol) for 5 minutes each and then washed for 5 minutes in water. Subsequently, staining with toluidine blue (0.5% toluidine blue and 1% sodium borate) was performed for 10 seconds, after the excess staining was removed with absolute ethanol, 70% ethanol and xylol 1 and 2 for 5 minutes each. Images of the articular surface of each sample were digitized and evaluated using Image J software (National Institute of Health, Bethesda). The result was expressed by the percentage of proteoglycan loss which is equivalent to the uncolored area in relation to the total surface area evaluated.

3.10 Isolation and purification of human neutrophils and mononuclear cells

Neutrophils were isolated from blood of healthy donors and mononuclear cells were isolated from human buffy coats using a Histopaque gradient. Two density-gradient separation media, Histopaque-1119 and Histopaque-1077 (Sigma-Aldrich, Inc.), were used to isolated leukocytes from whole blood. Histopaque-1119 and Histopaque-1077 and blood samples were added gently into a 15 mL centrifuge tube sequentially in a volume ratio of 1:1/2 and then centrifuged at 900 × *g* and 22 °C for 30 minutes. After centrifugation, granulocytes and monocytes were recovered from the Histopaque−1077/1119 and Histopaque-1077/plasma interfaces, respectively. Each layer was collected in a 15 mL centrifuge tube, the remaining Histopaque was washed out with PBS and enriched by centrifugation at 500 × *g* and 22 °C for 10 minutes. The cells were diluted to 1 % with PBS serving as the cell medium. The leukocytes were resuspended in Roswell Park Memorial Institute (RPMI) medium and counted in a Neubauer chamber.

3.11 Neutrophil cell culture and killing assay

Neutrophils were isolated from blood of healthy donors as described in the previous paragraph and were incubated with CXCL8 (1.25 µg/ml, 2.5 µg/ml or 5 µg/mL) or RPMI medium for 30 minutes before *S. aureus* was added at a multiplicity of infection (MOI) of 10:1 (bacteria:cell) for 3 hours. The number of surviving bacteria was determined after cell lyses with 1 % Triton X-100 for 10 minutes. Subsequently, serial dilutions of the lysate were prepared and incubated on agar plates overnight at 37 °C. Bacterial colonies were counted and expressed as CFU recovered.

3.12 Isolation and generation of human dendritic cells

Mononuclear cells were isolated from buffy coats using a Histopaque gradient as described before. CD14 Microbeads (Miltenyi Biotec) were added to the purified mononuclear cells and incubated for 15 minutes. The cells were washed and resuspended in buffer and added to a column in the magnetic separator. The unlabelled cells were washed from the column and the $CD14^+$ cells were eluted from the column by adding buffer solution. $CD14^+$ cells were counted and 5 x 10^6 cells were resuspended in 3 mL of complete medium supplemented with 100 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/mL of IL-4 and incubated at 37 °C and 5% CO_2. At day 3, new medium containing GM-CSF and IL-4 was added. At day 6, immature dendritic cells in the suspension were removed and activated by incubation for 24 hours with 20 ng/mL of LPS. Then, the cells were washed and counted in a Neubauer chamber.

3.13 Immunofluorescence assay

We performed the same procedure described above to recover cells from the synovial cavity 4 days after the infection for WT and 5-$LO^{-/-}$ and 7 days after the infection for vehicle and BOC2 treated mice. Cytospin slides were prepared from the joint wash using 3x10^5 cells and stained with the anti-CD11c phycoerythrin (PE) (1:10)

and anti-GR1 fluorescein isothiocyanate (FITC) (1:10) antibodies (BD Bioscience) and the nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI at 1mg/mL). The slides were fixed with acetone and the images were made using a confocal microscope (Nikon Eclipse Ti). Cells were quantified using the Volocity program and expressed as cells per field.

3.14 Flow Cytometry (FACs)

The popliteal lymph nodes of WT and 5-LO$^{-/-}$ animals infected with *S. aureus* were removed 4 days after the infection. A pool of 3 lymph nodes was used to represent a single sample. Lymph nodes were macerated and labeled with the following antibodies: CD11c FITC (1:10), anti MHCII PE Cy5 (1:300), CD86 Alexa 647 (1:100), CD4 V450 (1:100), CD3 allophycocyanin (APC) Cy7 (1:50) and isotype controls (1:100) (BD Bioscience). Cells were washed and fixed with 4 % formaldehyde and staining evaluated by flow cytometry (BD FACSCanto II). FlowJo software was used for analysis and the gate strategy is demonstrated in Figure 26.

3.15 Chemotaxis assays

The assay was performed using the Boyden chamber. Dendritic cells ($1x10^6$/mL) were placed in the upper compartment and allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents were present. CCL21 was used at concentrations of 10 ng/mL, 30 ng/mL and 100 ng/mL and LXA_4 at 100 ng/mL, 300 ng/mL and 1000 ng/mL. After 1,5 hours of incubation, the membrane between the two compartments was fixed and stained with May-Grunwald-Giemsa solutions and the number of cells that had migrated to the lower side of the membrane was determined by counting microscopically (magnification 500) in 10 high power fields/well. Chemotactic activity was expressed as the chemotactic index which is the mean number of cells migrated to the samples divided by the mean number of cells spontaneously migrated to the dilution buffer.

3.16 Statistical analyses

Data were expressed as mean ± standard of the means (SEM) and analysis performed using the statistical software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Differences between means or median 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.

4. RESULTS

4.1 A single intra-articular injection of Staphylococcus aureus causes prolonged joint inflammation, tissue damage and dysfunction

Septic arthritis caused by *S. aureus* infection is characterized by a massive influx of cells, mainly neutrophils into the affected joint (200). A single injection of *S. aureus* into the tibiofemoral joint of mice promoted intense accumulation of cells into the joint cavity in 24 hours that was sustained up to day 14. After 2 weeks, the total number of leukocytes started to decrease but significant numbers of leukocytes remained present at day 28 (Figure 7A). Neutrophil kinetics followed a similar profile as the total leukocyte numbers (Figure 7A and 7B) and neutrophils were the major cell type along all evaluated time points. The percentage of neutrophils among total leukocyte counts remained higher than 75 % up to day 7. The number of mononuclear cells increased later peaking at day 14 and decreasing thereafter (Figure 7C). The number of bacteria recovered from the infected joint was highest at day 1 and decreased thereafter. Of note, there was a significant number of bacteria even at day 28 after infection (Figure 7D). 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 (9). Here, mice infected with *S. aureus* presented an increase in histopathological score that peaked on day 7 after injection (Figure 8A, B). The first day of infection was characterized by a very marked influx of leukocytes, especially neutrophils, in the inflamed tissue. Intense cell infiltration persisted throughout the observation period, but there was a remarkable presence of synovial hyperplasia and bone reabsorption at later periods (Figure 8A, B). We evaluated the density of proteoglycans, which are important constituents of cartilage. Corroborating with the histopathological score, infected mice had a significant loss of proteoglycans at all times evaluated, and the most abundant loss at day 7 after infection (Figure 9A). Infected mice had continuous joint dysfunction, as assessed by measuring hypernociception, throughout the observation period of the study (Figure 9B). Thus, a single injection of *S. aureus* caused longstanding joint inflammation that was accompanied by significant tissue damage and pain. Since most evaluated parameters

peaked at day 7 after infection, this time point was evaluated in most subsequent experiments.

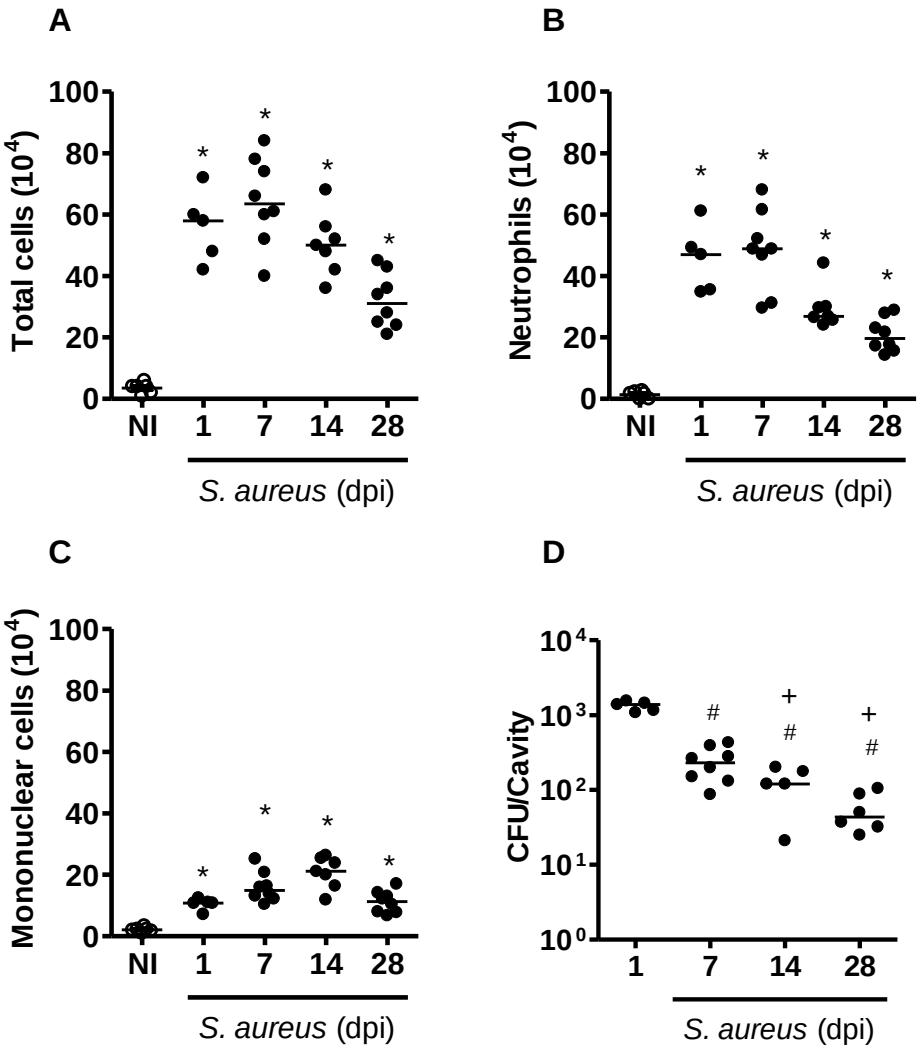

Figure 7 : Kinetics of cell recruitment and bacterial load in the joint after *S. aureus* infection. Mice were injected intra-articularly with *S. aureus* (10^7 CFU, 10 µL). Cells were harvested from the cavity 1, 7, 14 or 28 days after the injection. (A) The total number of leukocytes, (B) neutrophils 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 period. The data are representatives of three independent experiments. * $p< 0,05$ when compared with NI (non-infected) (ANOVA test followed by Newman Keuls' test) and # $p< 0,05$ when compared with 1dpi and + $p< 0,05$ when compared with 7 dpi (ANOVA test followed by Newman Keuls' test). N = 5-8 mice per group.

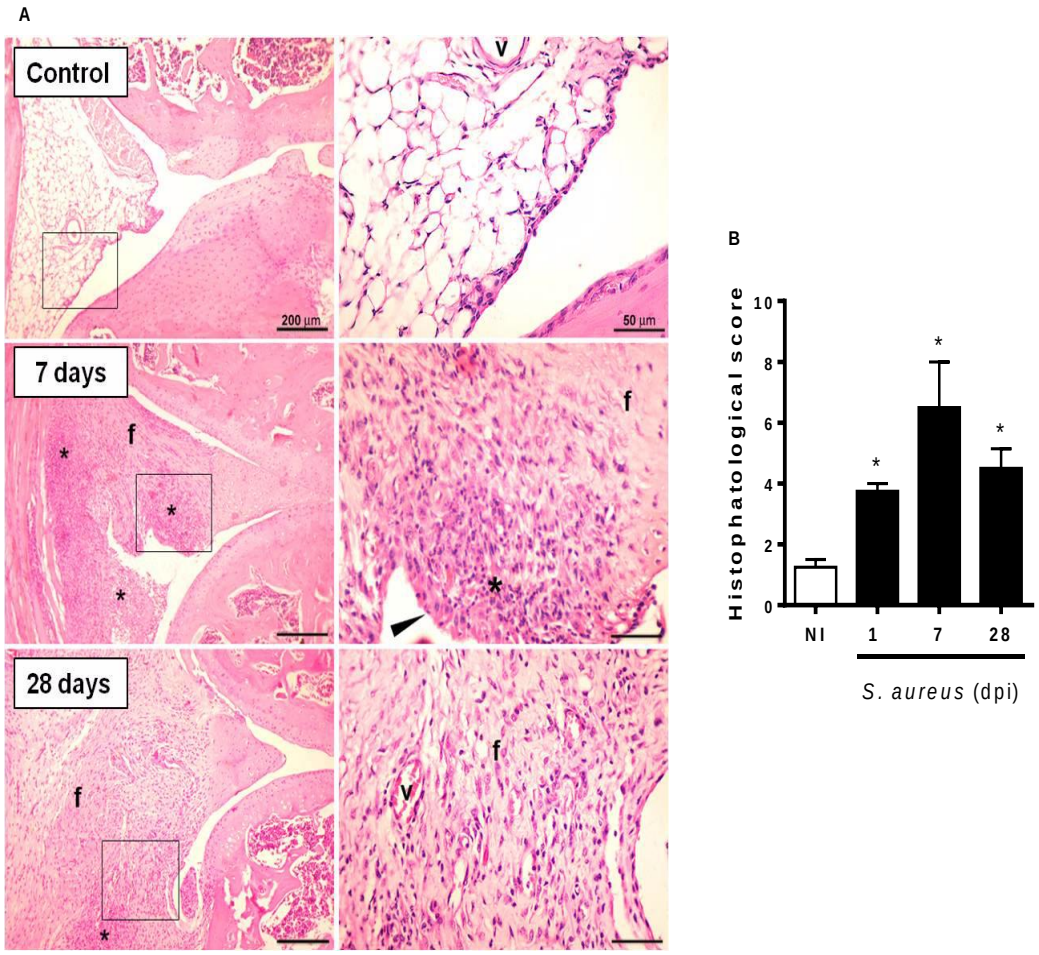

Figure 8: Kinetics of articular damage in *S. aureus*-induced arthritis. The joint was removed 1, 7 or 28 days after *S. aureus* infection and the tissue was processed for histopathological analyses. (A) Representative images of joints (v - blood vessels; f - collagen fibers; * - cellular infiltrate; arrowhead - synovial hyperplasia). (B) Histopathological score. The data show one representative out of two independent experiments. 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.

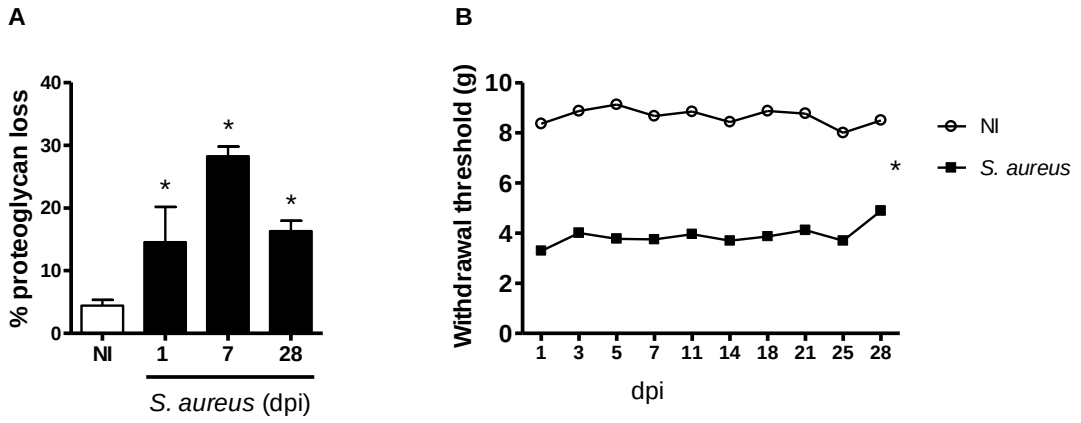

Figure 9: Kinetics of cartilage damage and hypernociception in *S. aureus*-induced arthritis. The joint was removed 1, 7 or 28 days after *S. aureus* infection and the tissue was processed for histopathological analyses. (A) % loss of proteoglycan was determined. (D) Hypernociception was evaluated before the euthanasia using an electronic analgesimeter at different time points following *S. aureus* injection. The data show one representative out of two independent experiments. dpi – days post infection. * $p < 0.05$ when compared to the non-infected (NI) group (ANOVA test followed by Newman Keuls' test for % loss of proteoglycan and T test followed by unpaired test for hypernociception). N = 3-5 mice per group.

Taken together, this set of results suggests a positive correlation between the presence of neutrophils and *S. aureus* in the infected joint which are associated with tissue damage and joint hypernociception. Considering the harmful effect of neutrophils in the joint causing arthritis (201), we hypothesized that the blockade of neutrophil recruitment could have a beneficial impact on the reduction of tissue damage and dysfunction following *S. aureus*-induced arthritis. Thus, we conducted different approaches targeting neutrophil-related chemotactic factors and organized this document in two chapters. In Chapter 1, we assembled the results based on the blockade of chemokine activities through the use of an antagonist of CXCR1/2 (202) and the use of a CXCL9-derived peptide that binds to GAGs and competes with GAG-chemokine interaction (159). In Chapter 2, we focus on the role of the 5-LO enzyme in *S. aureus*-induced arthritis, since an important metabolite of AA generated by this enzyme is LTB_4, a potent neutrophil chemoattractant. However, we gave special attention to another metabolite of this pathway, LXA_4, a renowned molecule for its anti-inflammatory effects.

4.2 Chapter 1: Blockade of chemokine functions in S. aureus-induced arthritis

4.2.1 The blockade of CXCR1/2 decreases neutrophil influx and ameliorates tissue inflammation, hypernociception and damage following S. aureus infection

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 those signs in non-infectious arthritis (203,204). In this study, we used the compound DF2156A, a non-competitive antagonist of CXCR1 and CXCR2, to impair the infiltration and activation of neutrophils in bacterially infected joints (202). In the first set of experiments, a group of mice were treated with DF2156A 1 hour before the injection of *S. aureus* and this treatment was repeated daily for the next 6 subsequent days. There was a reduction in the number of total leukocytes accumulated in joints of DF2156A-treated mice as compared to vehicle-treated control mice (Figure 10A). Importantly, there was a partial, but not a complete, blockade of neutrophil influx into the joint (Figure 10B). There was no reduction in the number of mononuclear cells recovered from the joint cavity (Figure 10C). At the molecular level, there was a marked reduction of CXCL1 production, a CXCR2 binding chemokine and neutrophil attractant, in inflamed tissue when compared to non-treated infected mice (Figure 10D). In addition, treatment with DF2156A led to the reduction of tumor necrosis factor (TNF) and interleukin (IL)-1β (Figure 10E, 10F). The amounts of chemokine and cytokines detected in the treated mice reduced to levels found in uninfected mice.

Treatment with DF2156A efficiently reduced tissue (Figure 11A) and cartilage (Figure 11B) damage. In addition, this treatment led to a reduction of the intensity of hypernociception, as seen by an increase of withdrawal threshold in the flexed joint (Figure 11C). 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.

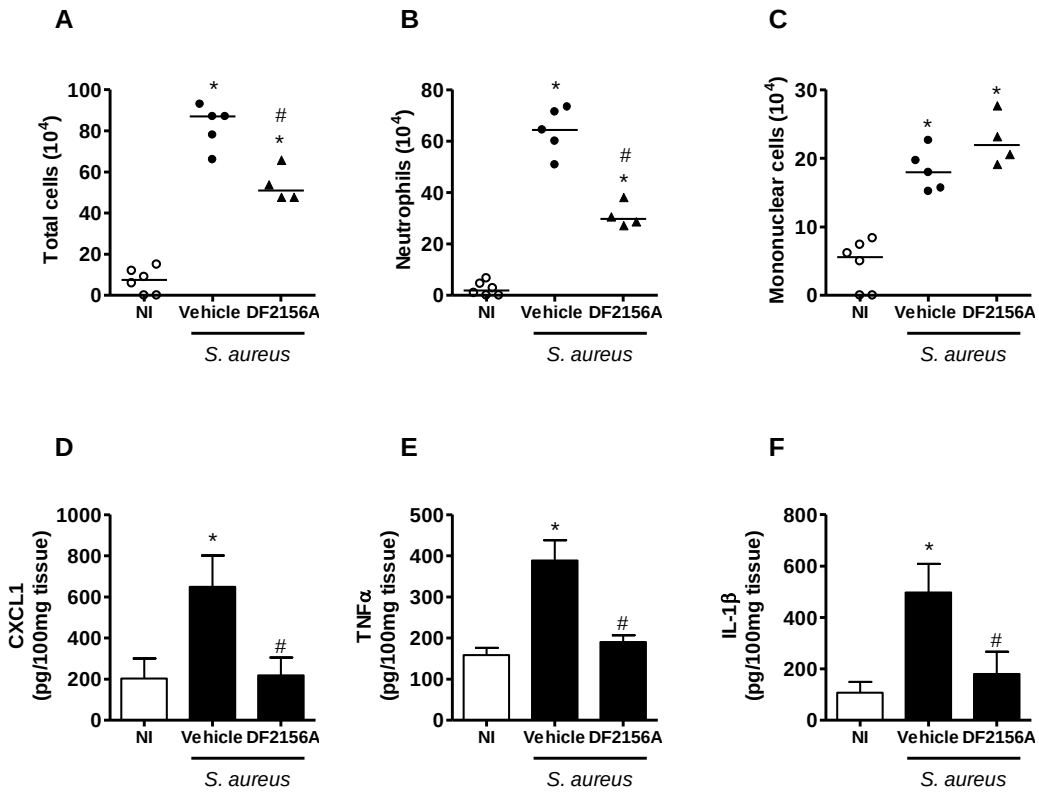

Figure 10: Effect of the treatment with DF2156A on cell recruitment and production of pro-inflammatory mediators in *S. aureus*-infected joints. Mice were infected with *S. aureus* or injected with sterile saline (NI – non-infected) into the tibiofemoral joint and the analysis was performed 7 days later. A group of mice were treated with DF2156A (DF – 10 mg/kg by gavage) 1 hour prior the injection of *S. aureus* and daily for the following 6 days. Migrated cells to the joint cavity were collected and counted as (A) the total number of leukocytes, (B) neutrophils and (C) mononuclear cells. The inflamed periarticular tissue was processed for the quantification of (D) CXCL1, (E) TNF and (F) IL-1β protein. The data show results from one representative out of three independent experiments. * $p < 0.05$ when compared with 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.

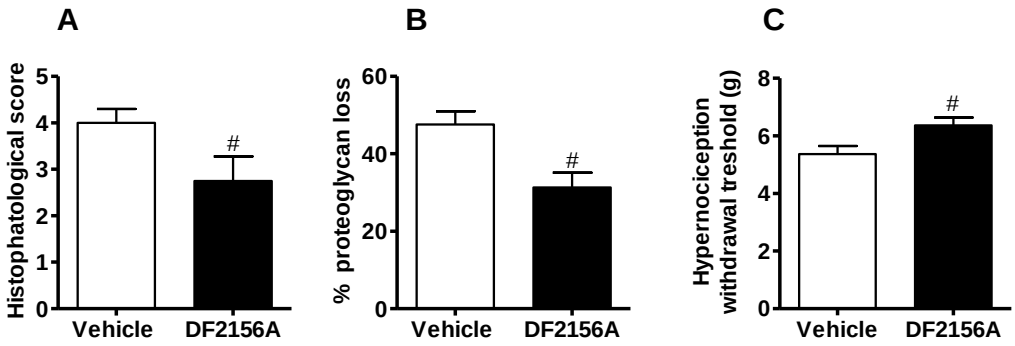

Figure 11: The effect of treatment with DF2156A on articular damage and hypernociception in *S. aureus*-infected joints. Mice were infected with *S. aureus* or injected with saline (NI – non-infected) into the tibiofemoral joint and the analysis was performed 7 days later. A group of mice was treated with DF2156A (DF – 10 mg/Kg by gavage) 1 hour 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. Whole joints were removed and processed for (B) histopathology and (C) the analysis of the loss of proteoglycan. The data represent one out of two independent experiments. # $p<0.05$ when compared to the vehicle-treated infected group (ANOVA test followed by Newman Keuls' test for histology; or T test followed by unpaired test for hypernociception). N = 3-10 mice per group.

4.2.2 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* in the joint. As seen in Figure 12A, the oral treatment with DF2156A from the very beginning of the infection impaired the clearance of bacteria. Next, we examined whether local treatment with DF2156A would affect the ability of the host to deal with the infection. As seen in Figure 7B, there was a significant neutrophil influx during the first days of the infection and we aimed to evaluate whether constant activation of neutrophils by CXCR1/2 acting chemokines was necessary for controlling bacterial replication. As shown in Figure 12B, local treatment with DF2156A impaired bacterial clearance. To confirm the importance of CXCR1/2 for clearance of the strain of *S. aureus* used in this study, we incubated human neutrophils with *S. aureus* in the presence or absence of different concentrations

of CXCL8, the most potent human ligand of CXCR1/2. Neutrophils alone partially control bacterial growth, but completely killed bacteria in the presence of CXCL8 (Figure 12 C). Although some chemokines have been reported to have direct antibacterial activity at elevated concentrations (205,206), CXCL8 alone, without neutrophils, at 5 µg/ml had no direct effect on the bacterial survival. 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.

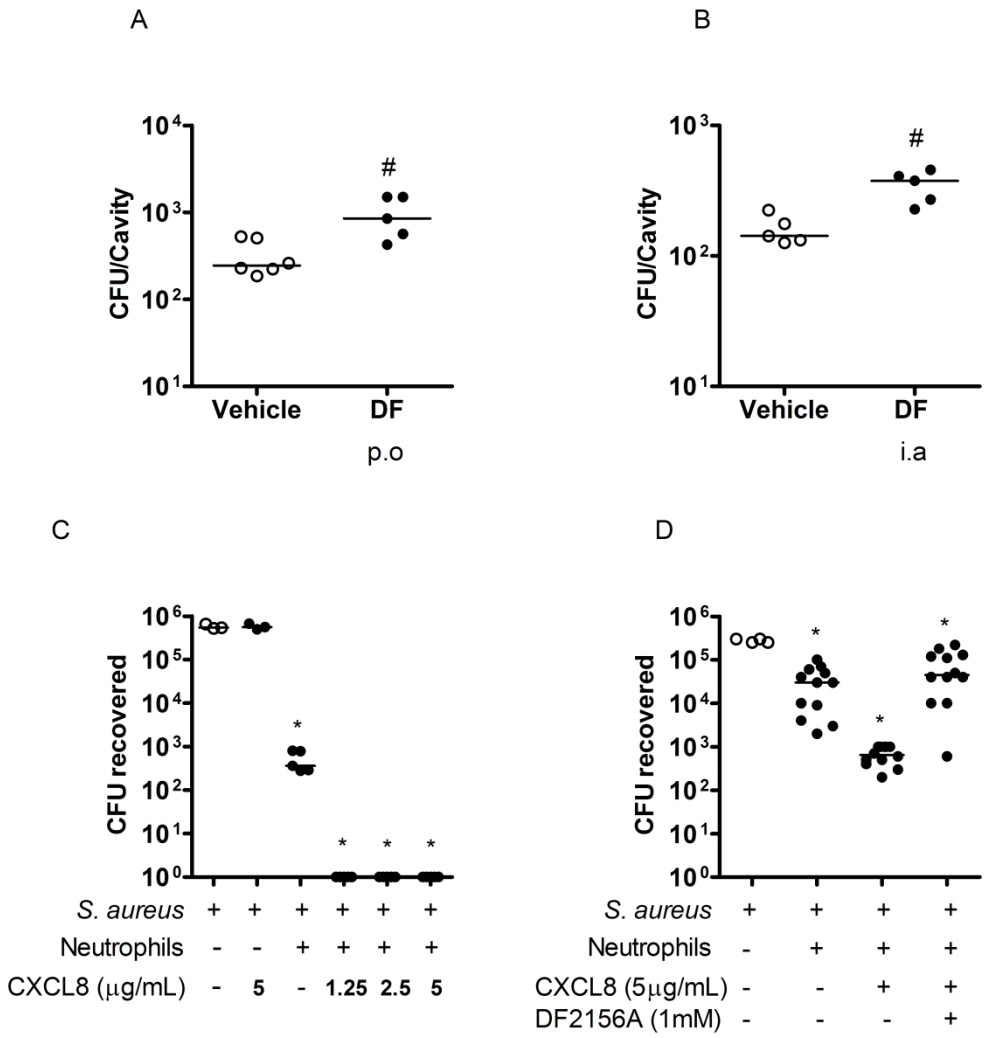

Figure 12: The effect of the treatment with DF 2156A in the control of *S. aureus*. Mice were infected with *S. aureus* into the tibiofemoral joint and the analysis was performed 7 days later. The inflamed periarticular tissue was collected for the analysis of the bacterial load. (A) A group of mice were treated with DF2156A (DF – gavage; 10 mg/kg) 1 hour prior the injection with *S. aureus* and daily for the following 6 days. (B) In another experimental approach, a group of mice was daily treated with DF2156A (intra-articularly; 10 μM) from day 3 to day 6 after the infection. (C and D) Peripheral blood human neutrophils were incubated with different concentrations of CXCL8 and DF2156A and infected with *S. aureus* at a MOI of 10:1 (bacteria:cell) for 3 hours for the analysis of bacterial killing. The data show one representative out of three independent experiments. # $p < 0.01$ when compared to the vehicle-treated infected group (T test followed by unpaired test). * $p < 0,05$ when compared with *S. aureus* or *S. aureus* + CXCL8 and + $p < 0,05$ when compared with S. *aureus* cultivated with neutrophils (ANOVA test followed by Newman Keuls test). N = 5-6 mice/ samples per group.

4.2.3 The treatment with DF2156A in a later stage of the disease decreases joint hypernociception and prevents the increase in bacterial load

The elapsed time between the infection and the first medication in septic arthritic patients is critical for the disease progression, including bacterial control, pain and joint damage (9). We showed that treatment with DF2156A from the start of the infection could prevent the main clinical parameters. However, this does not reflect the real clinical situation. Next, we started the treatment with DF2156A 3 days after the infection and evaluated parameters at different time points. Importantly, delayed treatment with DF2156A did not result in an increased bacterial load (Figure 13 A-C). Delayed treatment decreased joint pain early in the course of infection (day 4) but not at day 7 after infection (Figure 13D-F). However, there was no reduction on articular damage as assessed by histology as shown in Table 2.

Table 2: 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 (10 mg/kg, p.o.) 3 days after *S. aureus* injection and the tissue damage was evaluated at days 4 and day 7. Data are representative of three independent experiments. n =4-5 mice per group.

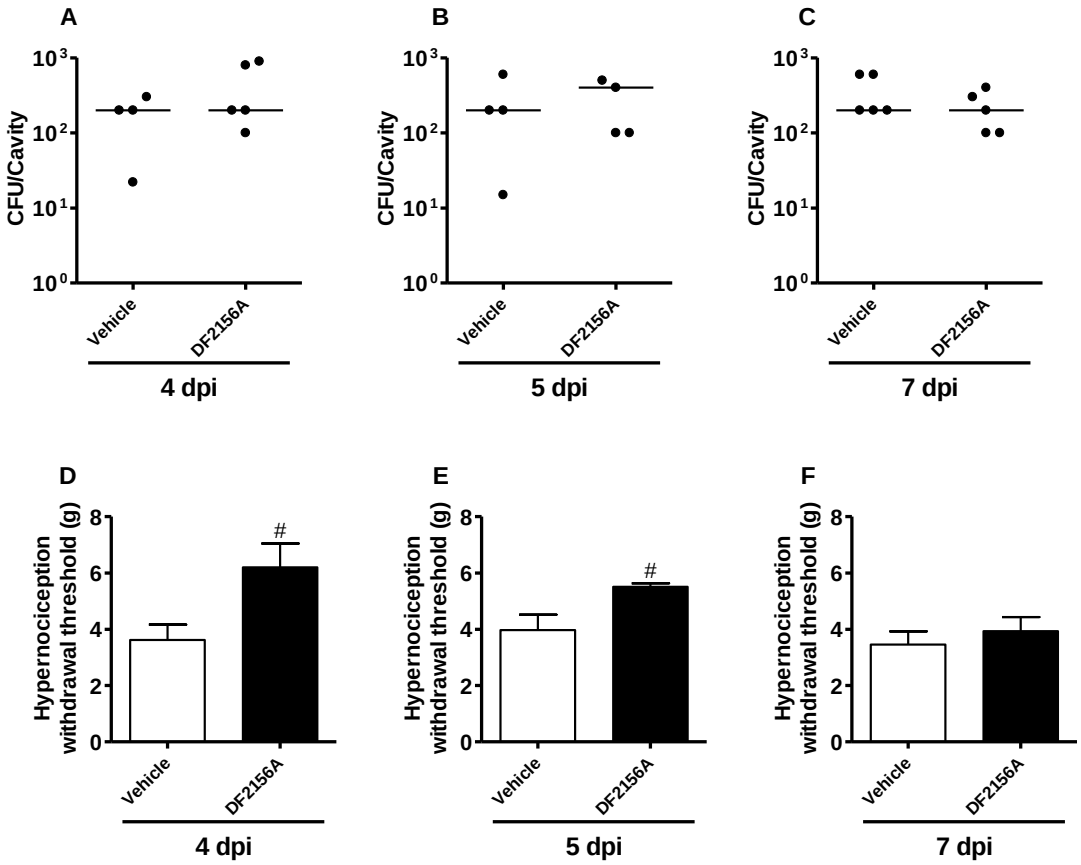

Figure 13: The treatment with DF2156A in a late stage decreases hypernociception and prevents the increase in bacterial load. Mice were infected with *S. aureus* into the tibiofemoral joint and treatment was started 3 days after infection (dpi). Different groups of mice were daily treated or not with DF2156A (DF – gavage; 10 mg/kg). The intensity of hypernociception was evaluated as the paw withdrawal threshold at days 4 (A), 5 (B) and 7 (C) after the infection. The inflamed periarticular tissue was collected for the analysis of bacterial load at the same time points (D, E and F). The data shown are from a representative of 2 experiments. # $p < 0.01$ when compared to the vehicle-treated infected group (T test followed by unpaired test). N = 4-5 mice per group.

In conclusion for this part, CXCR1/2 receptors contribute to the control of *S. aureus* replication in the context of septic arthritis. In addition, neutrophils recruited at the start of the infection have a major role in driving joint damage and dysfunction. The blockade of CXCR1/2 seems to 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.

4.2.4 The treatment with CXCL9 (74-103) inhibits neutrophil recruitment in peptidoglycan (PGN)-induced articular inflammation

In addition to CXCR1/2 blockade, we next aimed to investigate if a CXCL9-derived peptide, CXCL9(74-103), could reduce neutrophil recruitment and tissue inflammation after the injection of *S. aureus* or PGN, an important cell wall component of gram positive bacteria and "danger molecule", in mice. CXCL9 is an ELR^- CXC chemokine that consists of 103 amino acids and attracts T lymphocytes and NK cells. This chemokine has a long and basic C terminal region that ensures the interaction with GAGs (158). We chemically synthetized a C terminal portion of CXCL9 with 30 amino acids, CXCL9(74-103), and demonstrated previously that this peptide was able to compete with chemokines for GAG binding and consequently inhibited neutrophil recruitment in different non-infectious models of arthritis, such as monosodium urate crystal-induced gout and CXCL8-induced articular inflammation (159).

Initially, we investigated if the intra-articular injection of PGN would cause leukocyte recruitment to the synovial cavity. We used a dose of 1 μg/joint cavity according to previous unpublished data. As shown in Figure 14, PGN was able to recruit cells to the synovial cavity with a statistically significant difference from negative control mice at 6 and 24 hours after injection (Figure 14A). Most of the cells that were recovered from the joints at 6 hours post injection were neutrophils (Figure 14B). Neutrophil counts returned to basal level after 24 hours. On the other hand, the number of mononuclear cells in the joint only started to increase at 24 hours after the PGN injection (Figure 14C). Since the peak of neutrophils occurred 6 hours after PGN injection, we kept this time point for the next experiments.

Subsequently, we examined the effects of treatment with CXCL9 (74-103) on PGN-induced articular inflammation. In this set of experiments, mice received an intra-articular injection of PGN and were simultaneously treated with intravenously injected CXCL9 (74-103). The treatment was able to reduce the number of total cells (Figure 15A) and neutrophils (Figure 15B) that accumulated in the joints. However, the treatment had no effect on mononuclear cell recruitment (Figure 15C). We also evaluated chemokine production in the periarticular tissue following PGN injection and the potential effect of treatment with CXCL9 (74-103) on the amount of chemokines produced. The injection of PGN did not significantly alter the amount of any of the evaluated chemokines when compared to vehicle-injected joints, although there was a trend towards an increased concentration of CXCL1, CXCL2, CXCL10 and CCL3

(Figure 16). Thus, due to the high standard errors and limited number of mice tested, we could only detect a trend but no significant reduction of chemokine concentrations upon treatment of the mice with CXCL9 (74-103).

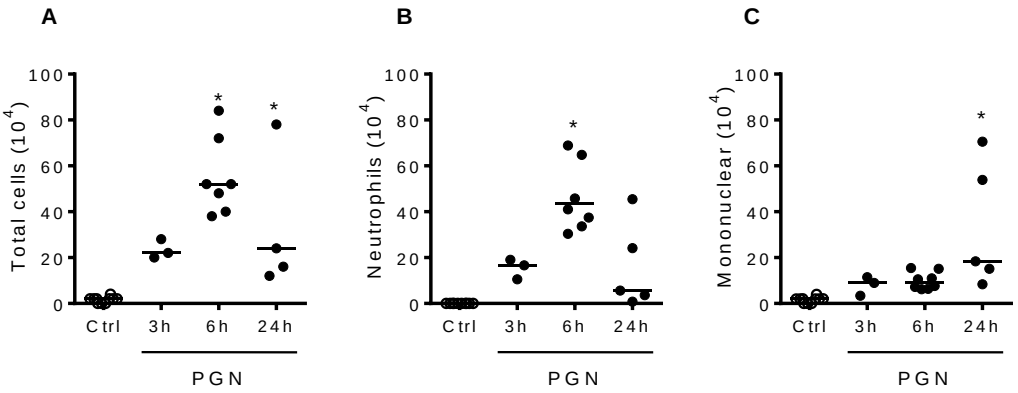

Figure 14: Kinetics of cell recruitment in peptidoglycan-induced articular inflammation. Mice received an injection of peptidoglycan (PGN) of 1 µg/10 uL or saline (control group - Ctrl) into the tibiofemoral joint and cells were harvested from the cavity 3 hours, 6 hours or 24 hours after the injection. (A) The total number of leukocytes, (B) neutrophils and (C) mononuclear cells recruited to the joint were counted under light microscopy in May-Grunwald-Giemsa -stained slides. The data are one representative of three independent experiments. * $p < 0.05$ when compared with the uninfected control group (ANOVA test followed by Newman Keuls' test). N = 3-6 mice per group.

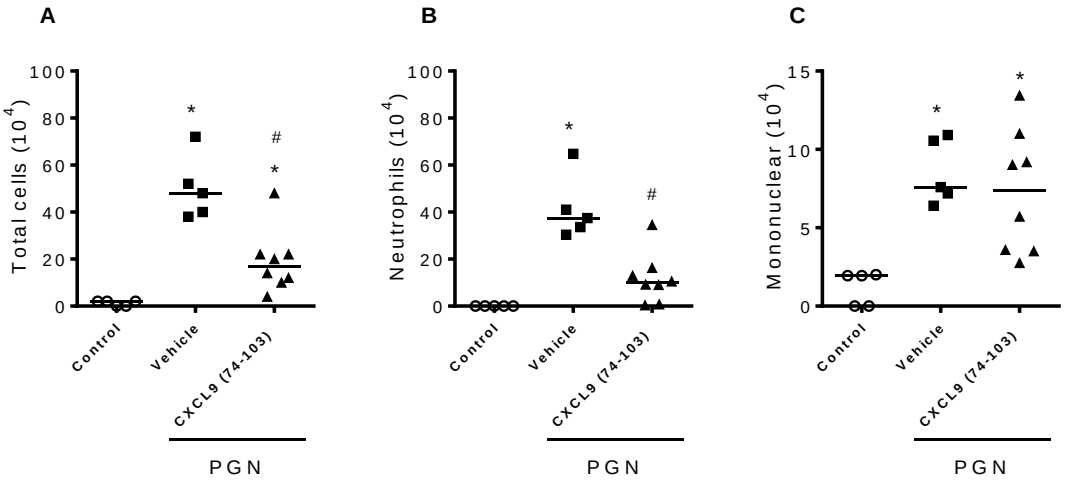

Figure 15: The effect of treatment with CXCL9 (74-103) on cell recruitment in peptidoglycan-induced articular inflammation. Mice received an intravenous (i.v.) injection into the tibiofemoral joint with peptidoglycan (PGN -1 μg/10 μL) or saline (control group). Simultaneously, a group of mice received an injection of CXCL9 (74-103) (100 μg/100 μL - i.v.) or saline (vehicle group). Cells were harvested from the cavity 6 hours after the injection. (A) The total number of leukocytes, (B) neutrophils and (C) mononuclear cells recruited to the joint were determined. The data shown are one representative of two independent experiments. * $p < 0.05$ when compared with the control group, # $p < 0.05$ when compared with the vehicle group (ANOVA test followed by Newman Keuls' test) N = 5-7 mice per group.

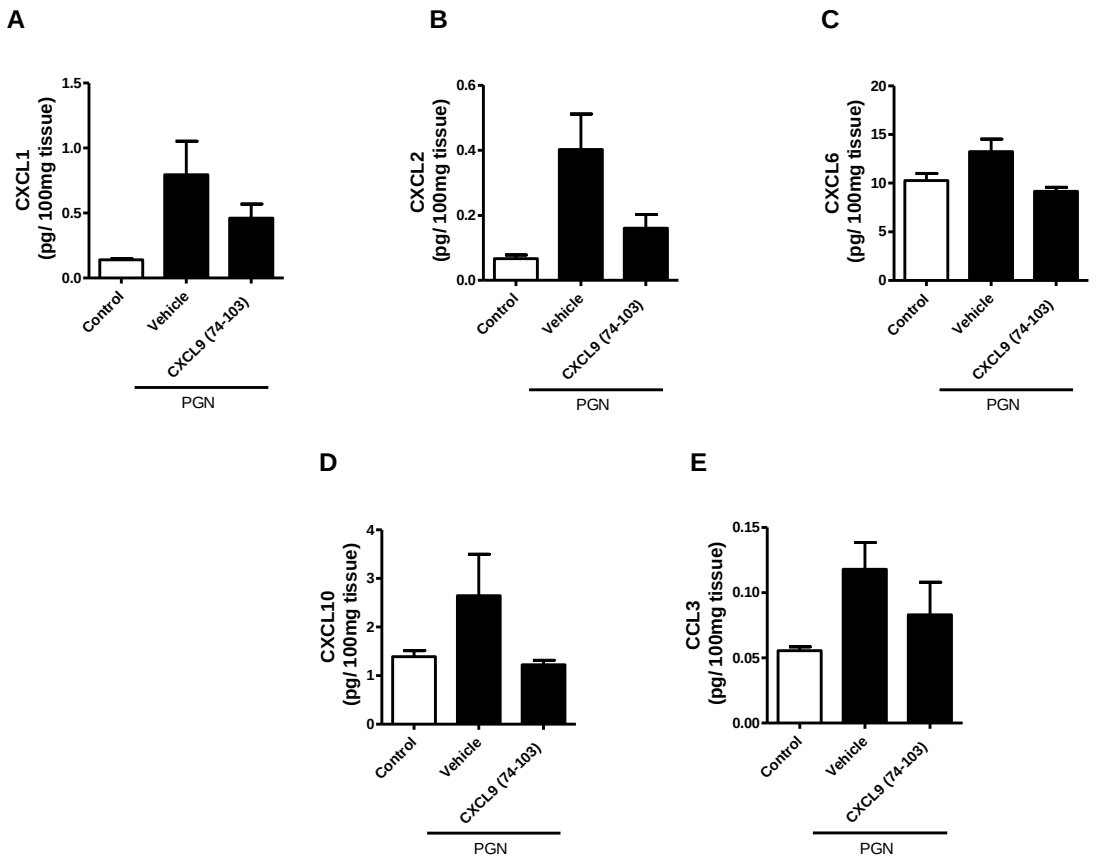

Figure 16: The effect of treatment with CXCL9 (74-103) on chemokine levels in peptidoglycan-induced articular inflammation. Mice received an intra-articular injection into the tibiofemoral joint with peptidoglycan (PGN) 1 μg/10 μL or saline (control group - Crtl). Simultaneously to PGN injection, mice were treated with CXCL9 (74-103) (100 μg/100 μL – i.v.) or saline (vehicle group – i.v.). The periarticular tissue was collected 6 hours after the treatment and processed for the quantification the chemokines: CXCL1 (A), CXCL2 (B), CXCL6 (C), CXCL10 (D) and CCL3 (E). N = 5-7 mice per group.

4.2.5 Treatment with CXCL9 (74-103) was able to decrease neutrophil recruitment in the septic arthritis model

Based on the aforementioned results of the effect of CXCL9 (74-103) in PGN-induced articular inflammation, we next investigated the effect of this treatment on *S. aureus*-induced arthritis. In this set of experiments, we infected mice with *S. aureus* and 3 days later we started the treatment with CXCL9 (74-103) every 12 hours until day 7.

In this treatment regimen, there was a significant reduction of accumulated total cells (Figure 17A) and neutrophils (Figure 17B) in the joint when compared to non-treated infected mice. On the other hand, the number of mononuclear cells (Figure 17C) was not affected by the treatment. The treatment was not able to reduce the level of the chemokines CXCL1 (Figure 17D), CXCL10 (Figure 17E) or CCL2 (Figure 17F).

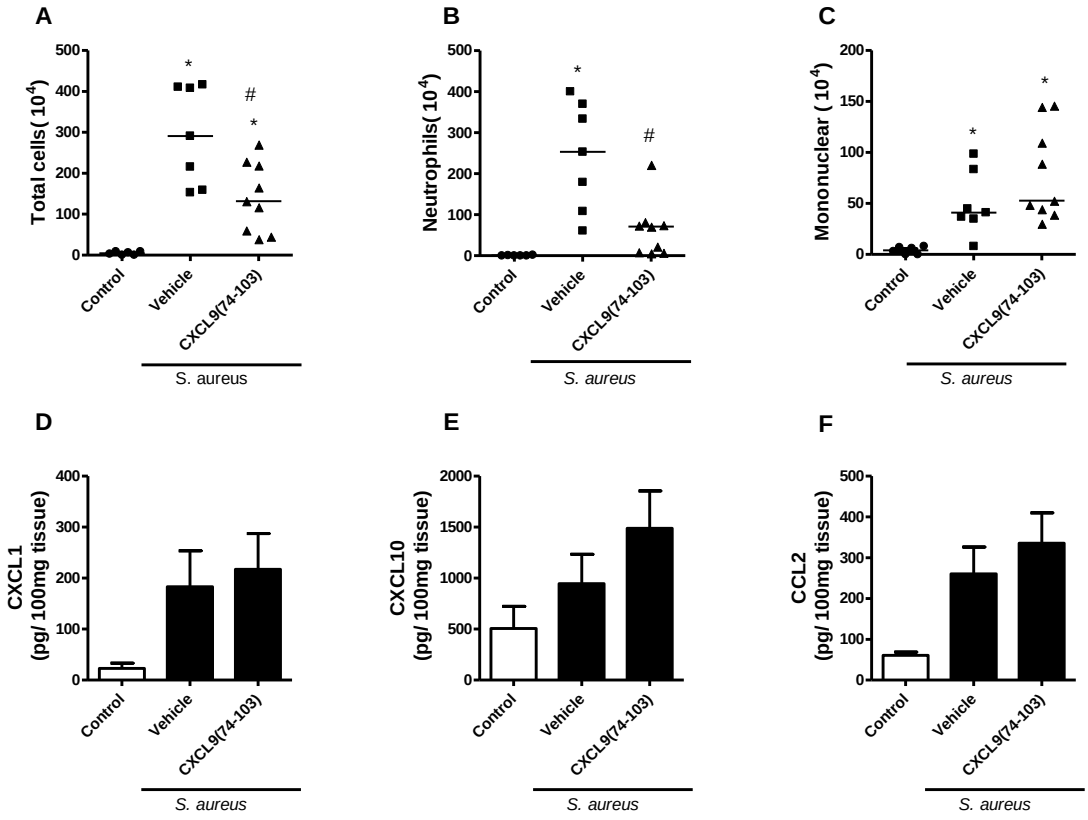

Figure 17: The effect of treatment with CXCL9 (74-103) on cell recruitment and chemokine production in *S. aureus*-induced arthritis. The mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non-infected) into the tibiofemoral joint. A group of mice were treated with CXCL9 (74-103) 100ug/100uL i.v. 3 days after the injection of *S. aureus* and each 12 hours for the following 4 days. Migrated cells to the joint cavity were collected and counted as (A) the total number of leukocytes, (B) neutrophils and (C) mononuclear cells. The periarticular tissue was collected and processed for the quantification of the chemokines CXCL1 (D), CXCL10 (E), and CCL2 (F). The data are from one representative of two independent experiments. * $p< 0.05$ when compared with non-infected control group; # $p< 0.05$ when compared to the vehicle-treated infected group (ANOVA test followed by Newman Keuls' test). N = 5-10 mice per group.

Next, we investigated if this treatment had an effect on the control of infection, tissue damage or joint hypernociception following *S. aureus* injection. The treatment with CXCL9 (74-103) increased the bacterial load recovered from the infected joint when compared to non-treated infected mice (Figure 18A). Furthermore, this treatment also increased the intensity of hypernociception (Figure 18B) and was not able to decrease the articular damage (Figure 18C) induced by *S. aureus*.

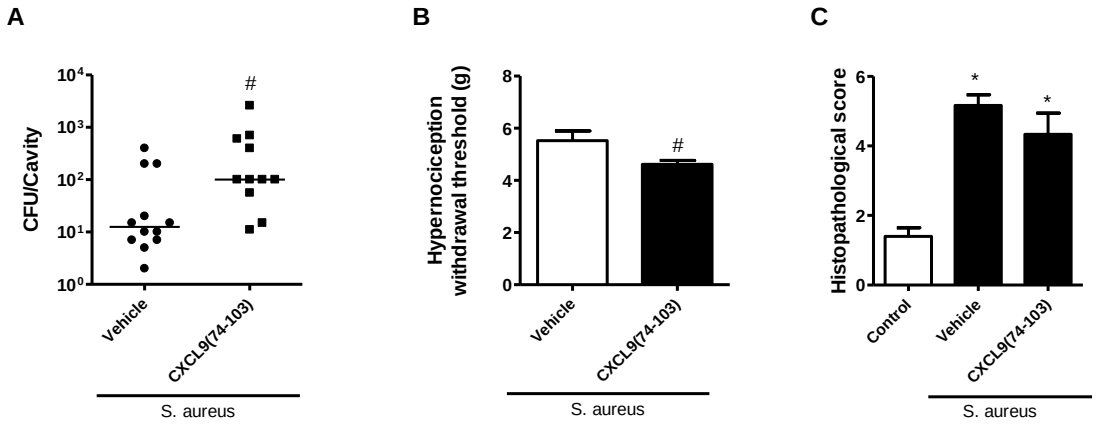

Figure 18: The effect of treatment with CXCL9 (74-103) on bacterial load, hypernociception and articular damage in *S. aureus*-induced arthritis. The mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non-infected) into the tibiofemoral joint. A group of mice were treated with CXCL9 (74-103) 100ug/100uL i.v. 3 days after the injection of *S. aureus* each 12 hours for the following 4 days. The inflamed periarticular tissue was collected for the analysis of bacterial load (A) and processed for histopathology (C). The intensity of hypernociception was evaluated as the paw withdrawal threshold (B). The data are one representative of two independent experiments. * p< 0.,05 when compared with the non-infected control group (ANOVA test followed by Newman Keuls' test). # p< 0.05 when compared to vehicle-treated infected group (T test followed by unpaired test). N = 5-10 mice per group.

In summary, the treatment with CXCL9 (74-103) was very effective in reducing neutrophil accumulation in the joint after injection with *S. aureus* or its cell wall component PGN. However, in the septic arthritis model, that therapy regimen did not demonstrate a satisfactory control of disease progression. Altogether, the results of CXCR1/2 blockade and CXCL9 (74-103) treatments could be useful to avoid an excessive inflammation, but need a carefully attention in their use during infections.

4.3 Chapter 2: The role of 5-Lipoxygenase during *S. aureus*-induced septic arthritis

4.3.1 The absence of 5-Lipoxygenase decreases tissue inflammation and improves the clinical manifestations of arthritis induced by *S. aureus*

5-Lipoxygenase (5-LO) is the main enzyme involved in the synthesis of LTB_4, an important chemoattractant for neutrophils. Thus, we next evaluated the role of 5-LO in *S. aureus*-induced arthritis. Initially, wild type (WT) mice and mice deficient for 5-LO (5-LO$^{-/-}$) were infected with *S. aureus* and inflammatory parameters evaluated 7 days after the infection. Both infected WT and 5-LO$^{-/-}$ mice presented an increase in number of total cells (Figure 19A) and neutrophils (Figure 19B) when compared to non-infected (NI) mice. Regarding the number of mononuclear cells, there was an increase of this number only in WT-infected mice when compared to its related NI group (Figure 19C). 5-LO$^{-/-}$-infected mice showed a significant reduction in number of cells recruited to the joints when compared to infected WT mice. Furthermore, the injection of *S. aureus* caused an increase of the concentration of CXCL1 (Figure 19D), CCL2 (Figure 19E) and IL-1β (Figure 19F) in periarticular tissue compared with NI mice. Infected 5-LO$^{-/-}$ mice had a significant reduction in these molecules when compared to infected WT mice.

Next, we investigated whether the absence of 5-LO would impact on articular damage, dysfunction or bacterial load. Infected WT mice presented an increased histopathological score when compared to NI mice (Figure 20). 5-LO$^{-/-}$ infected mice also had a higher histopathological score when compared to the related NI group, but had decreased articular damage compared to WT infected mice (Figure 20). We also analyzed cartilage damage by assessing the content of proteoglycan. 5-LO$^{-/-}$ mice had a reduced loss of proteoglycan following infection as compared to WT mice (Figure 21A), corroborating the histopathological score data. Furthermore, infected 5-LO$^{-/-}$ mice showed decreased hypernociception when compared to WT-infected mice (Figure 21B).

According to the data obtained until now, it is clear that 5-LO$^{-/-}$ mice have a substantial reduction in joint inflammation, damage and joint dysfunction upon *S. aureus* infection when compared to WT mice. Next, we analyzed if that reduced inflammation would impact on the bacterial control in the joint. Using a different strategy, we evaluated the bacterial load at different time points (1 day, 4 days and 7

days) after *S. aureus* injection. Both infected groups (WT and 5-LO$^{-/-}$) showed an equal bacterial load at 1 day and 4 days after the infection (Figure 21C). The amount of recovered bacteria started to decrease at day 7 in both groups. However, 5-LO$^{-/-}$ presented with even less bacteria in the joint when compared to WT mice indicating that they recovered faster from the bacterial infection.

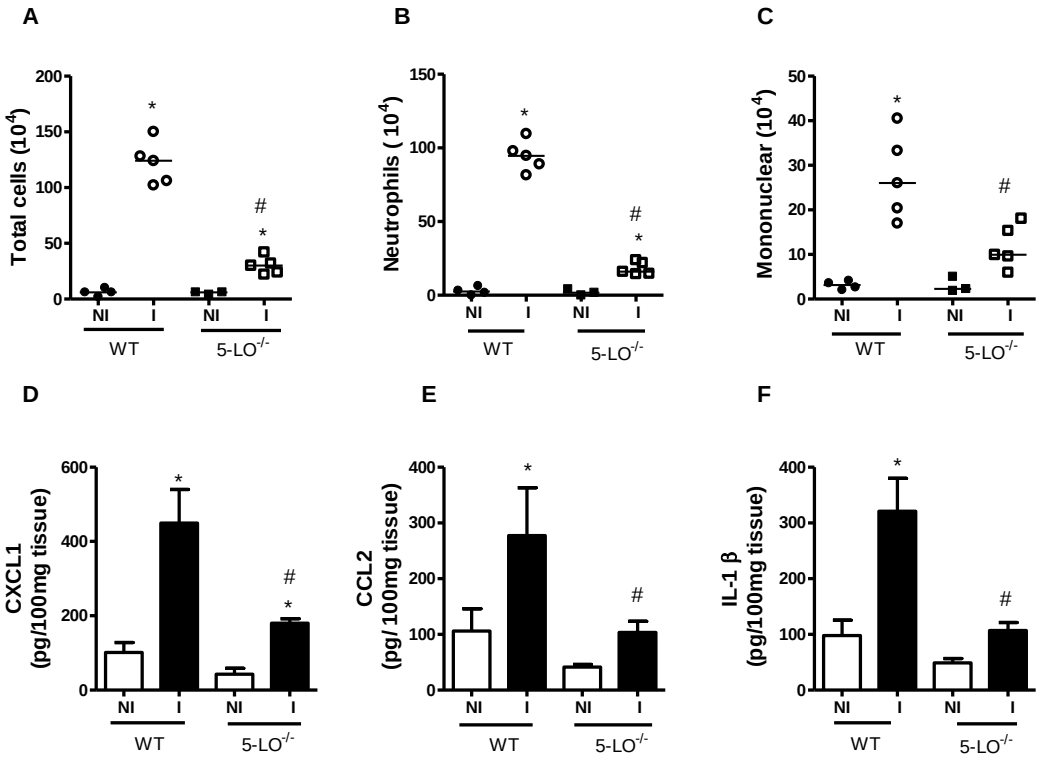

Figure 19: Cell recruitment and levels of inflammatory mediators in WT and 5-LO$^{-/-}$ mice in *S. aureus*-induced arthritis. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non-infected) into the tibiofemoral joint and the analysis was performed 7 days later. Migrated cells to the joint cavity were collected and counted as (A) the total number of leukocytes, (B) neutrophils and (C) mononuclear cells. The inflamed periarticular tissue was collected and processed for the quantification of the cytokine IL-1β (F) and the chemokines CXCL1 (D) and CCL2 (E). The data are one representative of three independent experiments. * $p < 0.05$ when compared with the NI group; # $p < 0.05$ when comparing the WT infected group with the 5-LO$^{-/-}$ infected group (ANOVA test followed by Newman Keuls' test). N = 5-6 mice per group.

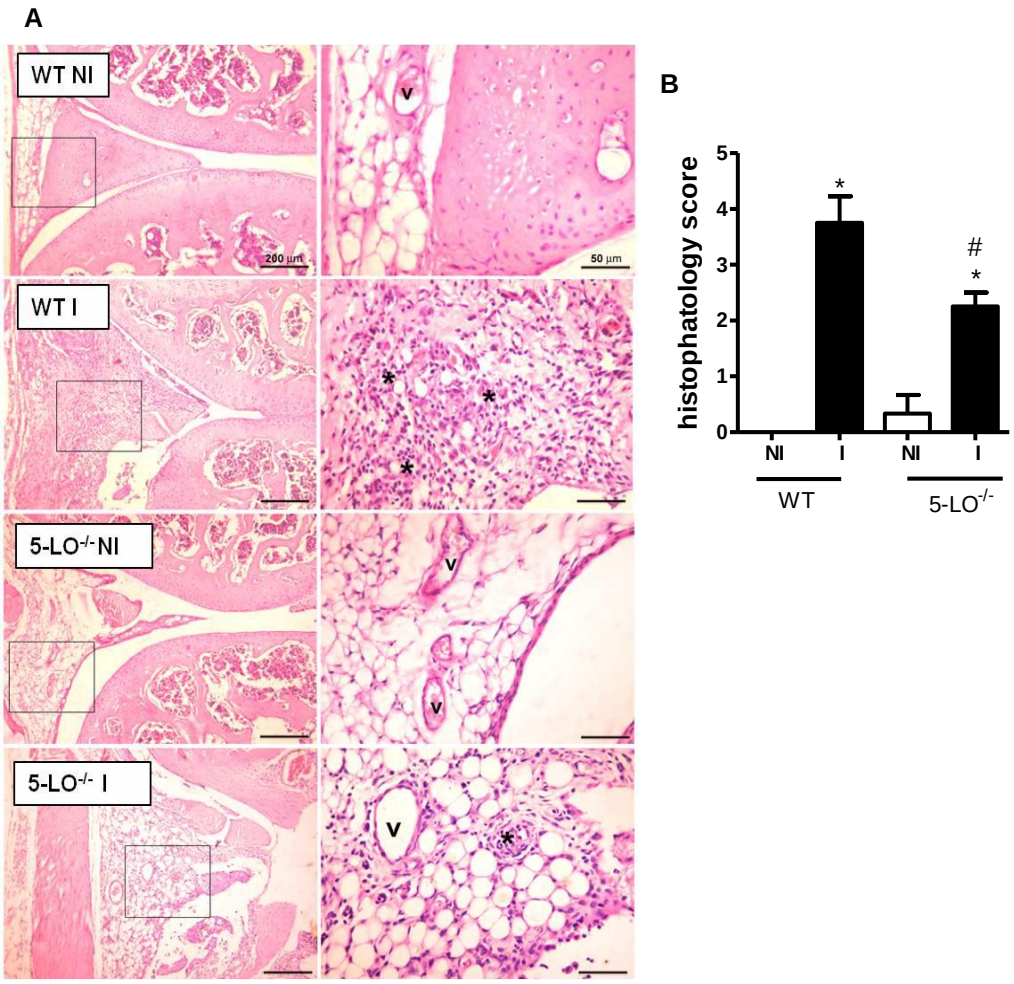

Figure 20: Articular damage in WT and 5-LO$^{-/-}$ mice in *S. aureus*-induced arthritis. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non infected) into the tibiofemoral joint. The joint was removed 7 days after *S. aureus* infection and the tissue was processed for histopathological analyses. (A) Representative images of joints (v - blood vessels; * - cellular infiltrate). (B) Histopathological score. The data show one representative out of two independent experiments. * p< 0.05 when compared to the non-infected (NI) group and # p<0,05 when comparing the WT infected group with the 5-LO$^{-/-}$ infected group. N = 3-5 mice per group.

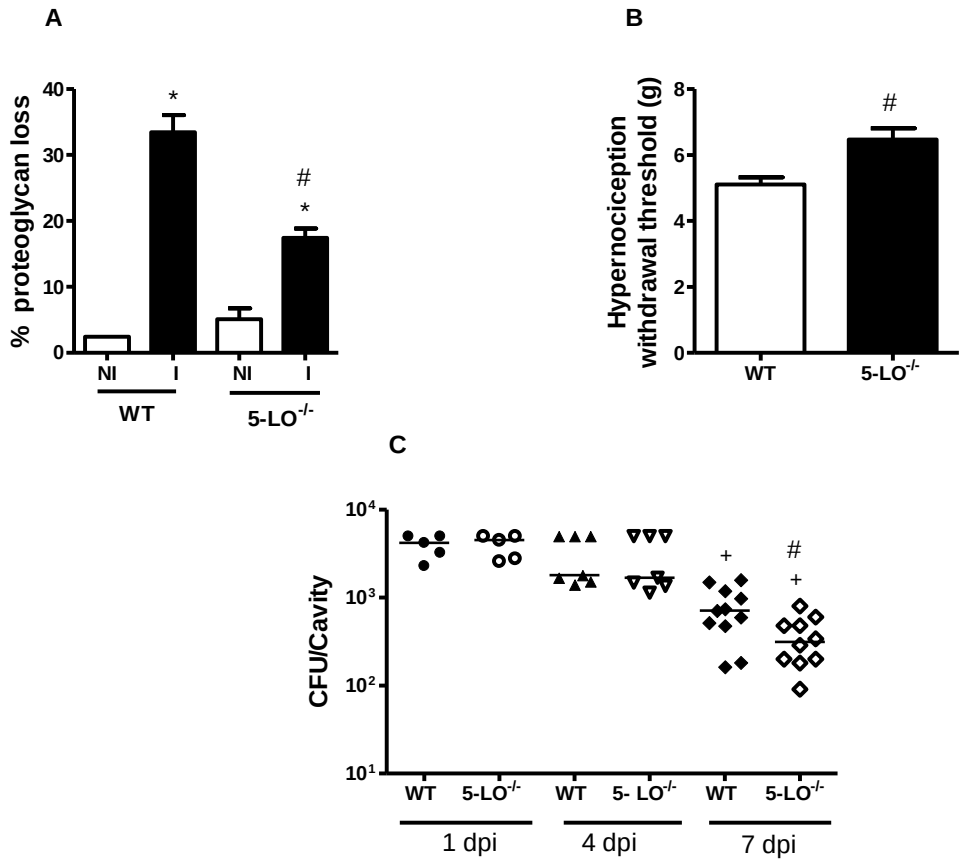

Figure 21: Evaluation of cartilage damage, hypernociception and bacterial load in WT and 5-LO$^{-/-}$ mice in *S. aureus*-induced arthritis. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non infected) into the tibiofemoral joint. The joint was removed 7 days after *S. aureus* infection and the tissue was processed for histopathological analyses. The cartilage damage is shown by the % loss of proteoglycan (A) and the intensity of hypernociception was evaluated as the paw withdrawal threshold (B). The inflamed articular tissue was removed for the analysis of the bacterial load at day 1, day 4 and day 7 (C). * $p< 0,05$ when comparing the WT infected with the WT NI or the 5-LO$^{-/-}$ infected with the 5-LO$^{-/-}$ NI mice, # $p< 0,05$ when comparing the WT infected group with the 5-LO$^{-/-}$ infected group (ANOVA test followed by Newman Keuls' test for bacterial load and T test followed by unpaired test for hypernociception). + $p< 0,05$ when comparing WT and 5-LO$^{-/-}$ mice at 4 dpi with WT and 5-LO$^{-/-}$ mice 7 dpi (ANOVA test followed by Newman Keuls' test). N= 5-10 mice per group

4.3.2 The levels of LTB_4 and LXA_4 in the joint are inverted along the kinetics of the infection

5-LO is a key enzyme to the production of lipid mediators such as leukotrienes and lipoxins from arachidonic acid. In order to investigate the mechanism that leads to the decrease in bacterial load in 5-$LO^{-/-}$ mice, we quantified the levels of LTB_4 and LXA_4 in periarticular tissue at different time points (1 day, 4 days and 7 days) after the *S. aureus* infection. The levels of LTB_4 were increased only in the first day of infection (Figure 22A) while LXA4 concentrations decreased at day 1, but kept at high levels at 4 days and 7 days after the infection (Figure 22B).

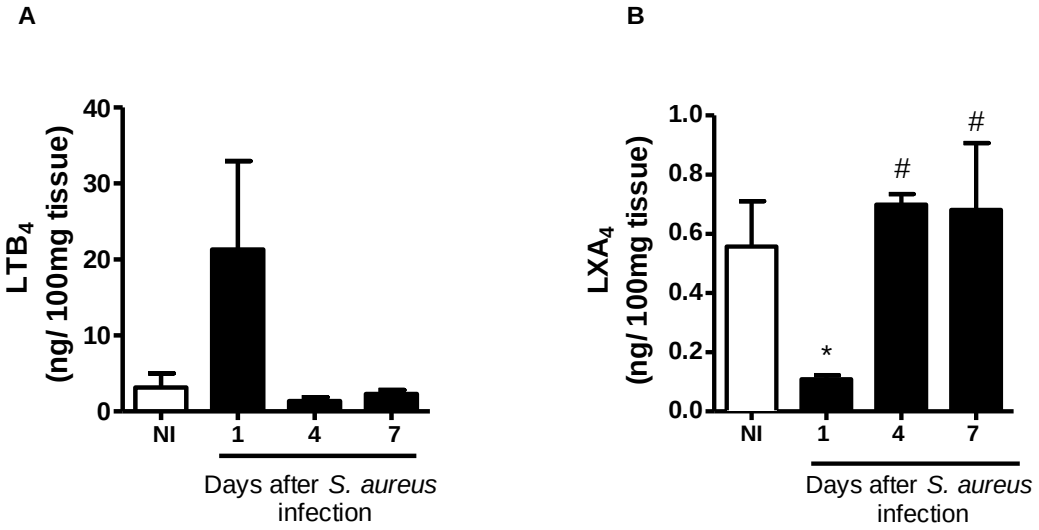

Figure 22: Level of LTB_4 and LXA_4 in periarticular tissue after injection with *S. aureus*. WT mice were infected with *S. aureus* (10^7 CFU/mL) or saline (NI – non infected) into the tibiofemoral joint. The joint was removed at 1 day, 4 days or 7 days after *S. aureus* infection and the tissue was processed for leukotriene B_4 (LTB_4) (A) and lipoxin A_4 (LXA_4) (B) measurement. * $p< 0,01$ when comparing with NI mice and # $p< 0,05$ when comparing mice with the 1 dpi group (ANOVA test followed by Newman Keuls' test) . N= 5-6 mice per group

4.3.3 The blockage of the LXA_4 receptor decreases cell recruitment and the bacterial load in the joint

Since the increased levels of LXA_4 in WT infected mice preceded the time at which 5-$LO^{-/-}$ mice started to have a reduced bacterial load and decreased numbers of leukocytes in the joint, we next investigated whether LXA_4 has a role in the control of inflammation and infection in *S. aureus*-induced septic arthritis. Lipoxin A_4 exerts its biological functions by binding to the ALX (FPR2) receptor. Thus, we first performed an experiment treating *S. aureus*-infected WT mice with an antagonist of both FPR1 and FPR2 (BOC2). The treatment was not able to decrease the total number of cells (Figure 23A) or mononuclear cells (Figure 23C) but significantly decreased the number of neutrophils (Figure 23B) as compared to non-treated-infected mice. Importantly, this treatment was also able to decrease the bacterial load when compared with the vehicle-treated group (Figure 23D). Next, we performed an experiment by treating infected WT mice locally with a specific antagonist of FPR2 (WRW4) and evaluated the bacterial load in the joint. The treatment with WRW4 was able to decrease the bacterial load (Figure 23E) similarly to BOC2 treatment. LXA_4 is not the only mediator that can bind to the FPR2 receptor. For instance the molecules Annexin A1 and serum amyloid A also signal through binding to FPR2 (207,208). Thus, in order to confirm that the effects of treatment with BOC2 or WRW4 depended on blocking LXA_4 activity, we daily injected LXA_4 locally in infected 5-$LO^{-/-}$ mice from day 4 (high levels of LXA_4 in WT mice - Figure 22) up to day 6 following infection. Such treatment with LXA_4 caused an increase in the articular bacterial load when compared to non-treated-infected mice (Figure 23F).

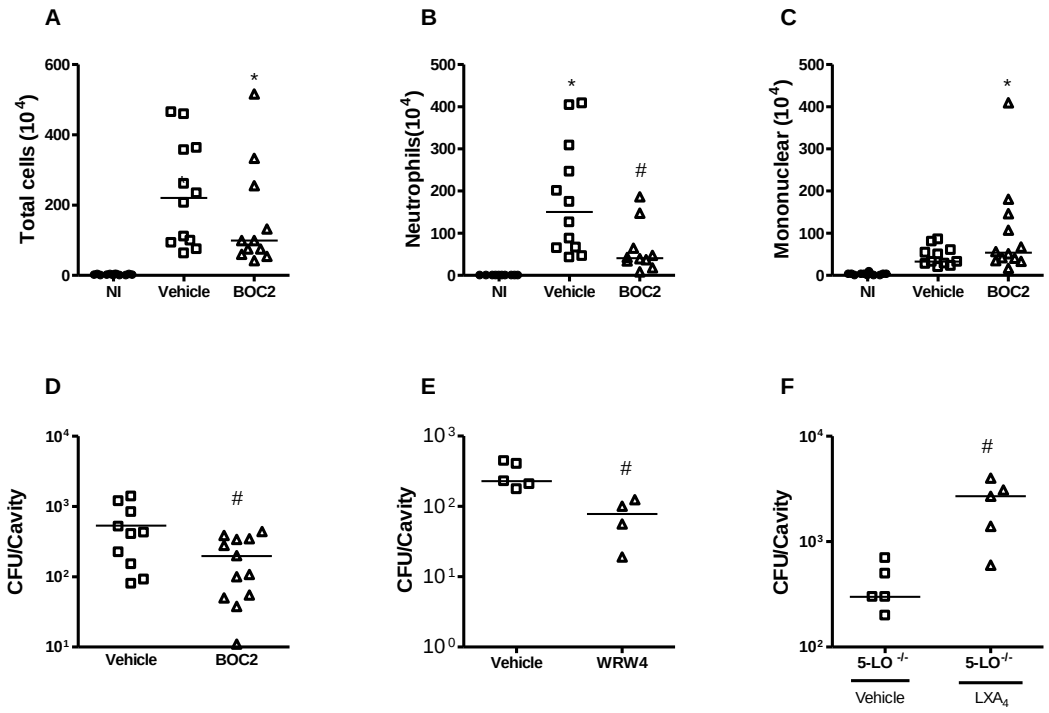

Figure 23: Effect of treatment with antagonists of the ALX receptor in cell recruitment and bacterial load induced by *S. aureus* injection. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or injected with saline (NI – non infected) into the tibiofemoral joint. One group of WT mice was treated with BOC2 (antagonist of both FPR1 and FPR2) at 2mg/kg intraperitoneally 4 days after the infection and daily until day 7. Cells that migrated to the joint cavity were collected and counted as (A) the total number of leukocytes, (B) neutrophils and (C) mononuclear cells. The inflamed periarticular tissue was collected for the analysis of the bacterial load (D). Another group of WT mice were treated intra-articularly with 10ug WRW4 (an FPR2 antagonist) in 10uL starting at day 4 after the infection and daily until day 7. The inflamed periarticular tissue was collected for the analysis of the bacterial load (E). One group of 5-LO$^{-/-}$ mice was treated intra-articularly with 20ng LXA_4 in 10uL starting at day 4 after the infection and daily until day 7. The inflamed periarticular tissue was collected for the analysis of the bacterial load (F). The data shown are from one representative out of three independent experiments. * $p< 0,05$ when compared with the NI group (ANOVA test followed by Newman Keuls' test) and # $p< 0.05$ when compared to the vehicle-treated infected group (T test followed by unpaired test) N = 4-12 mice per group.

4.3.4 The combined treatment with BOC2 and antibiotic tends to improve bacterial control and tissue damage after *S. aureus* infection

Since the antibiotic therapy is fundamental to control bacterial infections, we investigated whether the combination of BOC2 and vancomycin, a common antibiotic used in septic arthritis patients, could improve articular bacterial control and damage. Thus, mice were treated with BOC2 or vancomycin alone or in combination, starting 4 days after the infection. The individual treatments with BOC2 or vancomycin were able to reduce the bacterial load compared to the vehicle control (Figure 24A). However, the combined therapy completely eliminated the bacteria in the joint. All treatment regimens were able to decrease articular damage. Although there was a trend towards a further reduction of articular damage, there was no significant difference between the combined therapy and the isolated treatments with BOC2 or vancomycin (Figure 24B).

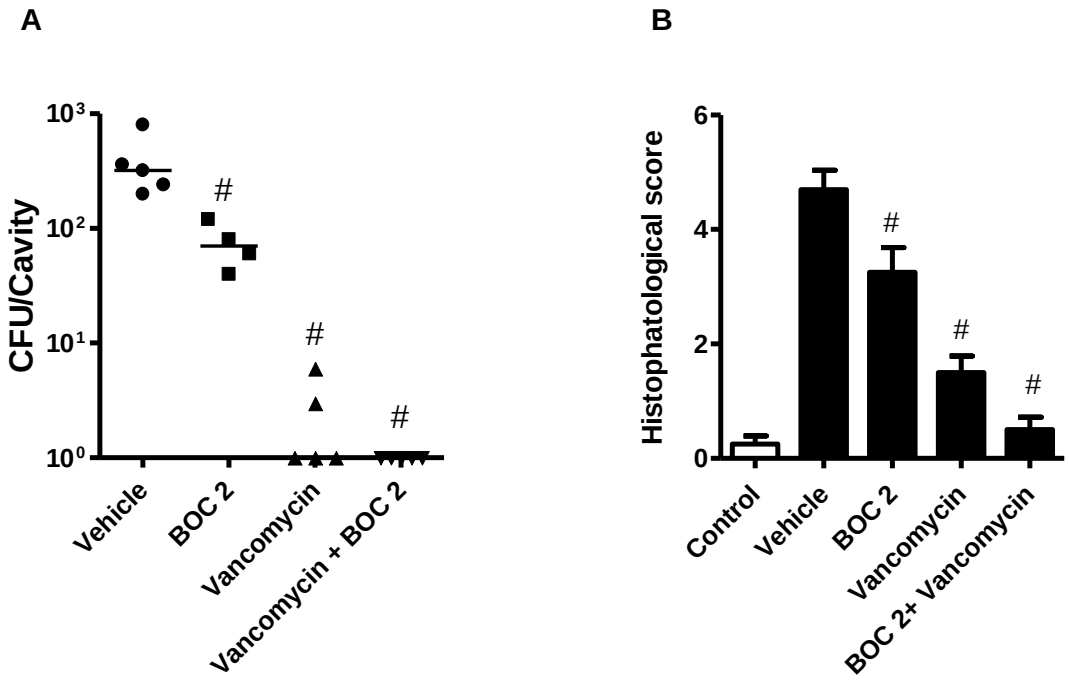

Figure 24: Effect of treatment with BOC2 and antibiotic on bacterial load and articular damage following *S. aureus* injection. WT mice were infected with *S. aureus* (10^7 CFU/mL) or injected with saline (NI – non infected) into the tibiofemoral joint. Four days after the infection, one group of mice was treated intraperitoneally with 2mg/kg BOC2 (antagonist of FPR1 and FPR2), another group was treated intraperitoneally with 400mg/kg of the antibiotic vancomycin and a third group of mice was treated with the combination of BOC2 and vancomycin. The mice received daily injections with BOC2, antibiotic or vehicle until day 7. The inflamed periarticular tissue was collected for the analysis of the bacterial load (A) or processed for histopathological analyses (B). # $p < 0.05$ when compared to the vehicle group (ANOVA test followed by Newman Keuls' test). N = 4-6 mice per group.

4.3.5 The absence of 5-LO and the blockade of FPR1 and FPR2 increase the number of $CD11c^+$ cells in the joint

In order to investigate the mechanism whereby LXA_4 could interfere with the control of the infection, we focused our attention on the presence and activation status of cells that have direct functions during the development of an adaptive immune response, such as dendritic cells and T cells. First, we evaluated the presence and role of

$CD11c^+$ (expressed on the surface of dendritic cells) during *S. aureus*-induced arthritis. $5\text{-}LO^{-/-}$ and WT mice treated with BOC2 (from day 4 to 6) were infected with *S. aureus* and cells were collected from the synovial cavity at day 7 after infection for immunofluorescence staining using antibodies against GR1 and CD11c. WT mice treated or not with the vehicle used to dilute BOC2 presented with higher amounts of $GR1^+$ cells compared to $5\text{-}LO^{-/-}$ and BOC2-treated WT mice (Figure 25A). On the other hand, those last two groups of mice presented with increased numbers of $CD11c^+$ cells (Figure 25 A-C). To investigate the role of $CD11c^+$ cells in the control of the bacterial infection, we performed an experiment in which we used CD11c DTR mice. In these animals DTR is under the control of a CD11c promoter and these mice allow for successful depletion of DC following a single injection of diphtheria toxin (DT). These mice were infected and 4 days after the infection we depleted the CD11c cells by injection of DT. The CD11c depleted mice (DT+) presented with an increase in the bacterial load compared to the non-depleted (DT-) mice (Figure 25D).

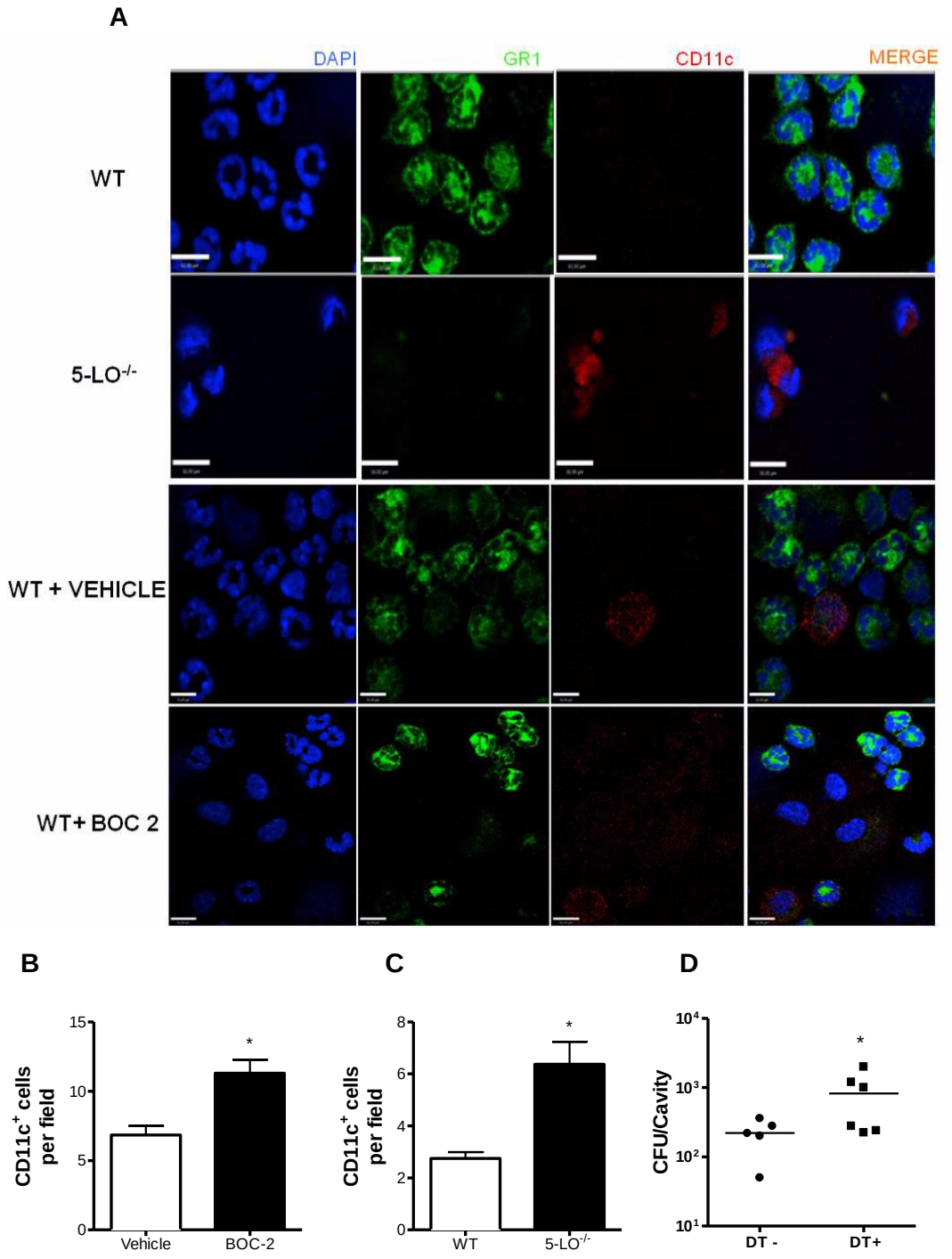

Figure 25: Presence of CD11c^{+} cells in the synovial cavity of WT, 5-LO$^{-/-}$ and BOC2-treated mice after *S. aureus* injection. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^{7} CFU/mL) or injected with saline (NI – non infected) into the tibiofemoral joint. One group of WT mice were treated with BOC2 (antagonist of FPR1/2 at 2mg/kg; i.p.) 4 days after the infection and daily up to day 6. Cells that migrated to the joint cavity were collected at day 4 for WT and 5-LO$^{-/-}$ groups and at day 7 for vehicle and BOC-2 treated mice. The cells were processed by immunofluorescence and stained for

CD11c and GR1. Representative images are shown in (A) and cells per field in (B) and (C). In another set of experiments, CD11c DTR mice were infected with *S. aureus* and 4 days after the infection CD11c cells were depleted (DT+) by intraperitoneal injection of diphtheria toxin (4ng/g). The inflamed periarticular tissue was collected for the analysis of the bacterial load (D) 7 days after the infection. * $p< 0,05$ N = 4-6 mice per group.

Next, we performed an experiment in which we infected WT and 5-LO$^{-/-}$ mice with *S. aureus* and 4 days later the popliteal lymph nodes were removed for cellular analysis. After the isolation procedure, cells were stained for CD11c, MHCII, CD86, CD3 and CD4 and analyzed by flow cytometry. The gating strategies are shown in Figure 26. Infected 5-LO$^{-/-}$ mice displayed with an increased number of CD11c^{+} cells (Figure 27A) compared to WT infected mice. Additionally, these CD11c^{+} cells seemed to be activated through the expression of MHCII (Figure 27B) and CD86 (Figure 27C). Furthermore, the number of CD3^{+} CD4^{+} T lymphocytes was also increased in 5-LO$^{-/-}$ when compared to WT mice (Figure 27D).

In order to confirm these results, we performed an experiment using mice that carry a fluorescent marker EYFP in CD11c^{+} cells (CD11c-EYFP). These mice were infected with *S. aureus* and one group received daily local injections with LXA_4 from day 4 to day 6. Seven days after the infection, the presence of CD11c^{+} cells in popliteal lymph nodes was evaluated by confocal microscopy. The treatment with LXA_4 was able to reduce the number of CD11c^{+} cells when compared to non-treated mice (Figure 28).

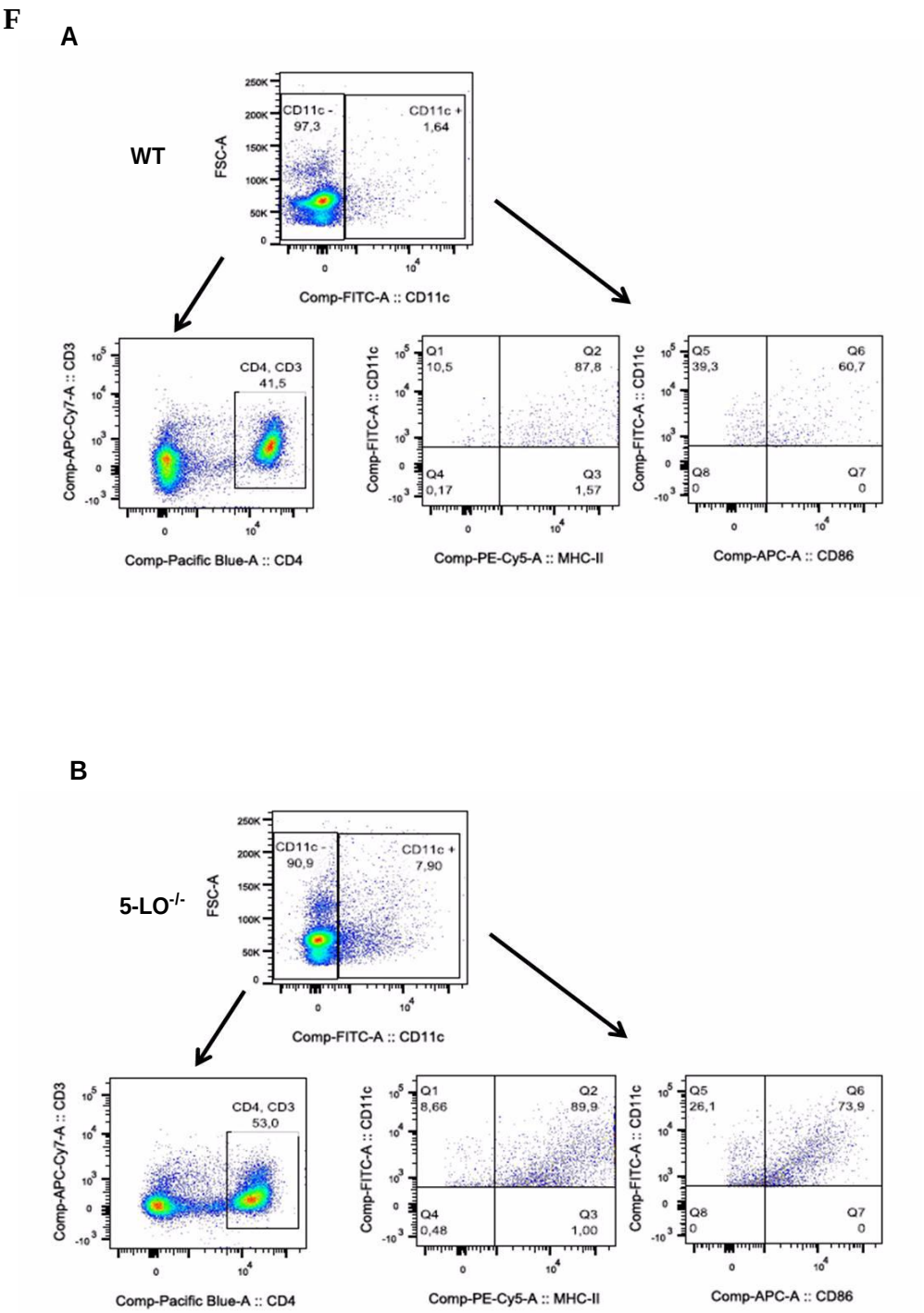

Figure 26: Gating strategy to evaluate the presence of CD11c cells and CD4 T cells in the lymph nodes. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or injected with saline (NI – non infected) into the tibiofemoral joint. The popliteal lymph nodes were removed 4 days after the injection, the cells were recovered and stained for CD11c, MHCII, CD86, CD3 and CD4 and analyzed by flow cytometry. (A) gating strategy for WT mice and (B) 5-LO$^{-/-}$. CD11c^{+} cells were gated and checked for MHCII and CD86, CD11c^{-} cells were gated and the presence of CD3 CD4+ cells was evaluated.

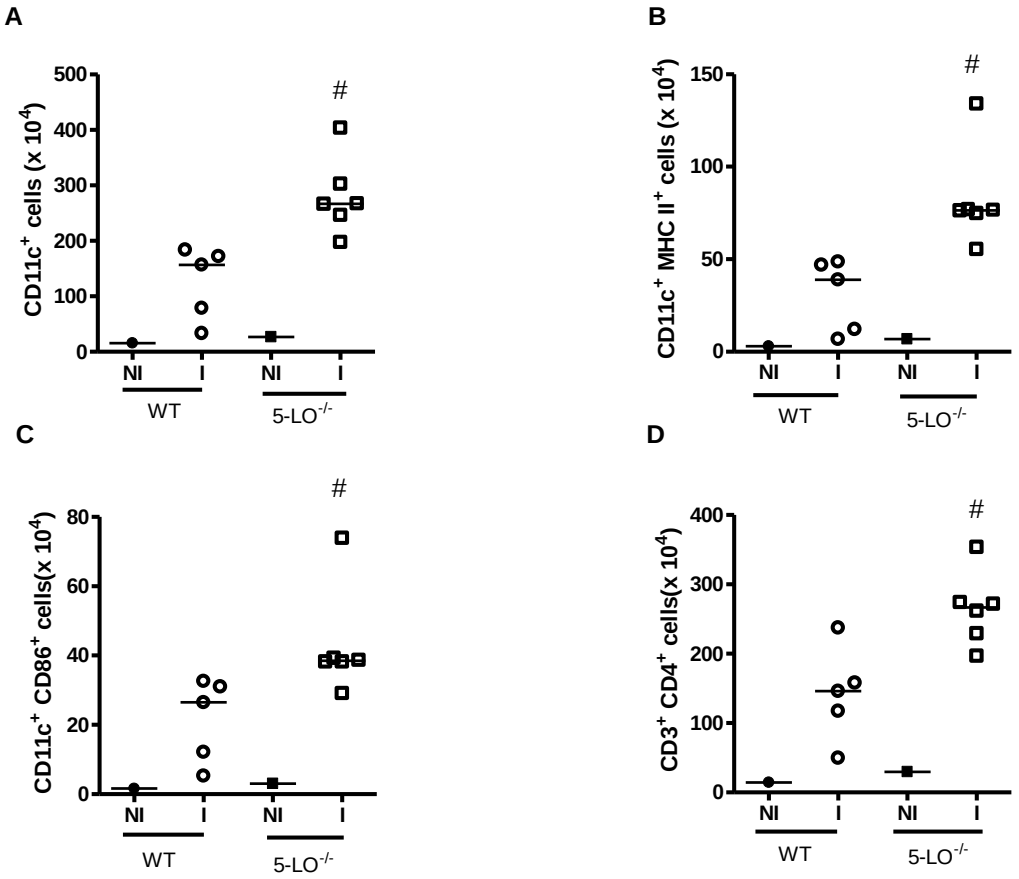

Figura 27: Detection of CD11c$^+$ and CD4$^+$ T cells in lymph nodes of WT and 5-LO$^{-/-}$ following *S. aureus* injection. WT and 5-LO$^{-/-}$ mice were infected with *S. aureus* (10^7 CFU/mL) or injected with saline (NI – non infected) into the tibiofemoral joint. The popliteal lymph nodes were removed 4 days after the injection, the cells were recovered and stained for CD11c, MHCII, CD86, CD3 and CD4 and analyzed by flow cytometry. The presence of CD11c$^+$ cells (A), CD11c MHCII $^+$ cells (B), CD11c CD86$^+$ cells (C) and CD3 CD4$^+$ cells (D) in lymph nodes was determined. # $p< 0.05$ when comparing WT infected mice with 5-LO$^{-/-}$ infected mice (T test followed by unpaired test). N = 4-6 mice per group.

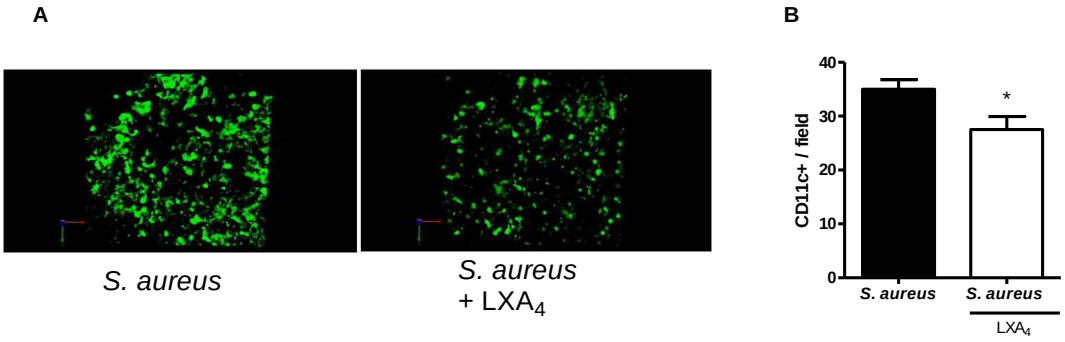

Figure 28: Presence of CD11c$^+$ cells in the lymph nodes after LXA_4 treatment in *S. aureus*-injected mice. CD11c-EYFP mice were infected with S. aureus (10^7 CFU/mL) or injected with saline (NI – non-infected) into the tibiofemoral joint. One group of mice were treated with LXA_4 (20ng/10uL) intra-articularly 4 days after the infection and daily during 7 days. The popliteal lymph nodes were removed and imaged by confocal microscopy (A) and CD11c$^+$ cells were counted per field (B). * $p < 0.05$ when comparing LXA_4 treated mice with non-treated mice (T test followed by unpaired test). N = 4-5 mice per group.

4.3.6 The treatment with LXA_4 inhibits human dendritic cell chemotaxis in response to CCL21 *in vitro*

Since the absence of 5-LO and blockade of FPR1 and FPR2 increased the number of CD11c$^+$ cells in the lymph nodes and joints, we next investigated whether LXA_4 could have a direct role on the chemotaxis of dendritic cells. It is well know that the migration of dendritic cells to lymph nodes is dependent on the chemokines CCL19 and CCL21 through binding to CCR7 (209). Thus, using a boyden chamber chemotaxis assay, we evaluated the chemotaxis of purified human dendritic cells in response to CCL21 in the presence or absence of different concentrations of LXA_4. LXA_4 inhibited in a dose dependent manner the recruitment of dendritic cell recruitment (Figure 29). Altogether, these results indicate that LXA_4 impairs the control of *S. aureus* in this model, causing prolonged joint inflammation and dysfunction.

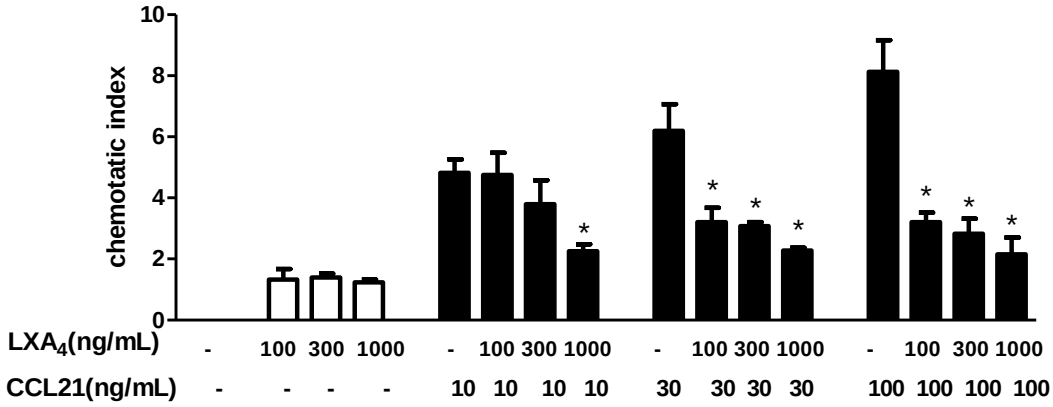

Figure 29: Chemotaxis of human dendritic cell in response to CCL21 after LXA_4 treatment. Human dendritic cells were incubated or not with different concentrations of LXA_4 (100, 300, 1000 ng/mL) and their chemotactic response to different concentrations of CCL21 (10, 30, 100 ng/mL) (A) was determined in a Boyden chamber chemotaxis assay. * $p< 0.05$ when comparing the chemotactic index for dendritic cells with or without treatment with LXA_4 for the different CCL21 concentrations (ANOVA test followed by Newman Keuls).

5. DISCUSSION

Neutrophils have a crucial role in controlling bacterial infections. They use different effector mechanisms that are fundamental for bacterial killing, such as phagocytosis, synthesis of antimicrobial peptides, release of their granular content including a number of lytic enzymes, production of reactive oxygen and nitrogen species, and release of their nuclear content creating the neutrophil extracellular traps (NETs) (210). Here we showed that the intra-articular injection of *S. aureus* causes a massive recruitment of neutrophils to the joint and the presence of these cells correlated with the bacterial clearance. Similar data were observed with depletion of neutrophils in an experimental model of arthritis induced by hematogenous injection of *S. aureus*, causing an increase in bacterial burden (211). Nonetheless, the effector mechanisms used by neutrophils are also associated with tissue damage and pain in infectious and non-infectious arthritis and other diseases. During sepsis, high concentrations of cell-free DNA/NETs were found in patients and their presence was associated with organ dysfunction (212). Also patients with rheumatoid arthritis produce more NETs in circulation and higher amounts of ROS than those in unaffected individuals (213) and the presence of these mediators is related with an increased inflammatory response and joint damage (214). Furthermore, the presence of neutrophils is related to increased pain under different types of stimulation such as injection of carrageenan (215), zymosan (216) or methylated albumin in antigen-induced arthritis (217). A simple injection in the joint of neutrophil elastase, a protease present in neutrophil granules and an important protease in the generation of NETs, causes hypernociception in mice (218). In accordance, we also showed that the presence of neutrophils in the joint is related to articular damage and hypernociception. Thus, the control of *S. aureus*-induced arthritis must be very well regulated, avoiding bacterial spreading but also excessive tissue damage and dysfunction.

The initial fundamental therapy for septic arthritis patients is the use of antibiotics combined with a joint drainage procedure. However, even with this treatment, about 25-50% of patients present joint dysfunction or permanent tissue damage. Thus, the use of antibiotics combined with anti-inflammatory molecules could impact on reduction of articular damage and pain. Two clinical trials in children tested the use of dexamethasone combined with antibiotics, they showed that such treatment can reduce the symptoms, articular damage and hospitalization time (219,220). Similar

tests in adults are not available. In the mice model of septic arthritis, the combination of an inhibitor of TNF with antibiotics (221) and bisphosphonate combined with antibiotics and corticosteroids (222) were able to decrease bone resorption and tissue damage. However, it needs to be carefully applied due to the risks for uncontrolled bacterial growth or spreading. For instance, the depletion of neutrophils by antibodies has been tested during *Toxoplasma gondii* (223), *Listeria monocytogenes* (224) and *Staphylococcus aureus* (225) infections and in all cases the bacterial burden was increased.

Recently, the demonstration that neutrophils have a role in the pathogenesis of arthritis and cancer made them targets for therapies (226,227). In an antigen-induced arthritis model, the pharmacological inhibition of CCR2 or the use of $CCR2^{-/-}$ mice showed a reduction in the recruitment of neutrophils and in inflammation (228). The inhibition of the PI3K pathway, a downstream signaling pathway of chemokine receptor activation, in collagen-induced arthritis decreased inflammation and the reduced damage correlated with the decrease in neutrophil migration (229). In this thesis, we demonstrated that neutrophils are important for articular damage and hypernociception. Thus, we hypothesized that the inhibition of neutrophils could be beneficial to decrease these clinical parameters. Therefore, a combined therapy including antibiotics and anti-neutrophil compounds could be effective and needs more studies

Chemokines are important chemoattractants for leukocytes. Their effects in cellular recruitment are dependent on chemokine immobilization on the endothelial surface by binding to GAGs, facilitating their interaction with their specific GPCRs on leukocytes to exert their functions. Since the 90's, when chemokine receptors were characterized and later when it was demonstrated that HIV entry into the cell depended on binding to the chemokine receptor CCR5, special attention was dedicated to chemokine receptors for the development of new drugs (230,231). However, to date only two small molecules have reached the market: Maraviroc (Pfizer) a CCR5 inhibitor for HIV infection (232) and Plerixafor (Anormed) a CXCR4 inhibitor for stem cell mobilization during chemotherapy (233). The earlier blockade of CXCR1 and CXCR2 by antagonists has shown to be beneficial in some articular diseases. The treatment with the antagonist Reparixin or DF2162 in an antigen-induced arthritis model decreased neutrophil recruitment, inhibited TNF production, hypernociception, and the overall severity of the disease in the tissue (234). In another arthritis model, anti-collagen antibody-induced arthritis, the treatment with SCH563705, a potent small molecule

antagonist of CXCR1/2, showed a decrease in clinical disease scores, paw thickness, reduced inflammation, bone and cartilage degradation (204). Another antagonist, DF2755A was able to reduce inflammatory hyperalgesia induced by carrageenan, LPS or the murine chemokine CXCL1/KC. In addition, the antagonist inhibited neutrophil recruitment and also reduced post-incisional nociception (235). Our results corroborate with these findings since we observed a decrease in neutrophil recruitment, inflammatory mediators, articular damage and hypernociception.

Neutrophils are necessary for *S. aureus* elimination during infection (236). We showed that the earlier treatment with a dual CXCR1 and CXCR2 antagonist decreased neutrophil influx, but increased, as expected, the bacterial load. The blockade of CXCR1/2 showed similar results with other bacteria such as *Nocardia asteroids* (237) and *Pseudomonas aeruginosa* (238) in lung infection. So, the treatment that starts in the early phase of the infection is effective in the control of articular damage and hypernociception by decreasing neutrophil infiltration, but fails to control the infection. Trying to translate these observations to a clinical situation, we started the treatment with DF2156A in a late stage of the disease. However, such later treatment was not able to significantly decrease the articular damage, although it transiently decreased hypernociception and prevented the increase in bacterial load. Thus, we propose that the initial neutrophil infiltration is sufficient to cause articular damage. The combination of the CXCR1/2 antagonist and antibiotics needs to be investigated to investigate the potential clinical benefits of such treatment.

Recently, a new option to target the chemokine system was the observation that some modified chemokines can antagonize intact chemokines (239). We used a CXCL9-derived peptide that has been demonstrated to inhibit neutrophil recruitment in different articular models by binding to GAGs and competing with chemokines for GAG binding (158,159). Since the effect of treatment with CXCL9 (74-103) during infection was not accessed before, we first used a simple model based on the injection of peptidoglycan, a “danger molecule” present in the *S. aureus* cell wall. As we had previously shown in non-infectious articular diseases such as experimental gout, the treatment with CXCL9 (74-103) was able to inhibit neutrophil recruitment induced by peptidoglycan. Next, we tested a comparable treatment in *S. aureus*-induced septic arthritis. In contrast to CXCR1/2 blockade, the treatment with CXCL9 (74-103) was not able to decrease hypernociception or the bacterial load. The blockade of CXCR1/2 decreased the levels and activity of CXCL1. It was demonstrated that the injection of

CXCL1 into the joint caused an increase of mechanical hypernociception in mice by a mechanism dependent on the synthesis of prostaglandins and sympathetic amines, well-known nociceptive stimuli (240). Additionally, it was shown that *S. aureus* can directly activate nociceptive neurons (241) that could explain the increase in hypernociception.

Leukotriene B_4 plays a key role in inflammation, mainly due to its potent effect on neutrophil recruitment. Clinical trials using LTB_4 antagonists for treatment of rheumatoid arthritis and lung diseases in patients with cystic fibrosis have been performed (242,243). Since leukotrienes are produced in a pathway dependent on 5-lipoxygenase, we initially used knockout mice for 5-lipoxygenase to study the role of LTB_4 in our model. We noticed a decrease in neutrophil and mononuclear cell infiltration, inflammatory mediators and hypernociception in the infected knockout mice. However, in contrast to experiments with the chemokine receptor antagonist, the decrease in number of neutrophils did not correlate with an increase in bacterial load in 5-$LO^{-/-}$ mice. Conversely, the 5-$LO^{-/-}$ mice had a diminished bacterial load compared to WT mice. To investigate better the role of 5-LO in the bacterial control, we measured the levels of lipid mediators produced in the enzyme pathway, i.e. LTB_4 and LXA_4. Increased LXA_4 was found at the same time point that bacterial counts decreased. LXA_4 is a lipid mediator involved in the resolution of inflammation and its *in vivo* functions are the opposite of the role of LTB_4 (244). LXA_4 can signal through binding to the ALX/FPR2 receptor. However, LXA_4 is not the only mediator that binds to FPR2. Annexin A1, a renowned pro-resolving molecule and glucocorticoid-derived protein (208), and serum amyloid A (245) have also been described to bind to the same receptor. It is important to make it clear that even though LXA4 is important in the resolution of inflammation, our aim here is to demonstrate that the blockade of LXA_4 is beneficial for bacterial control. To demonstrate that the effect on bacterial control was mediated by LXA_4, we treated WT mice with 2 different antagonists of the FPR2 receptor. One of them, BOC2, is not a selective antagonist, since it can block both FPR1 and FPR2. The treatment with this antagonist had similar results as infection experiments performed with 5-$LO^{-/-}$ mice, also decreasing the bacterial load. Another antagonist, WRW4, selectively blocks FPR2 and had a similar effect, decreasing the bacterial load after *S. aureus* infection. Although, the blockade of LXA_4 receptors is not sufficient to demonstrate that the effect is mediated by the lipid, since other mediators also bind to the same receptor, we treated 5-$LO^{-/-}$ mice with LXA_4 and observed the reversion of the phenotype, i.e. a decrease of the capacity to control the infection.

These results are opposite to observations described in the literature for some infections. LXA_4 has been demonstrated to be important for bacterial control and survival in sepsis (246), infection with *Pseudomonas aeruginosa* (192) and *Toxoplasma gondii* (247). The treatment with 15-epi-LXA4, an analogue derived from aspirin, showed to be beneficial in *Trypanosoma cruzi* infection (248). In *Mycobacterium tuberculosis* infection, it has been demonstrated that 5-$LO^{-/-}$ mice presented with lower bacterial burden in the lungs and the administration of a LXA_4 analog in 5-$LO^{-/-}$ mice preserved the protection. This indicates that lipoxins, and not leukotrienes, negatively regulate host control of *Mycobacterium tuberculosis* infection (249). In contrast, other studies have reported that inhibition of 5-LO with the pharmacological inhibitor MK886 abrogated host control of experimental pulmonary tuberculosis and that this was associated with a reduction in leukotrienes (250). Despite the discrepant outcomes between experiments in 5-$LO^{-/-}$ mice and MK886-treated mice, the finding that 5-LO variants are associated with susceptibility to tuberculosis supports that 5-LO products, lipoxin A4 and leukotrienes, regulate the host-protective immune response against *Mycobacterium tuberculosis* (251). In accordance with our data, it has been demonstrated that during infection with *Brucella abortus*, 5-$LO^{-/-}$ mice also display a reduction in bacterial load in spleen and liver (252).

In order to investigate the mechanism of blockade of LXA_4 that leads to control of the infection, we hypothesized that LXA_4 could increase the recruitment or activation of a specific cellular type, such as macrophages. However, we found an increased number of activated $CD11c^+$ cells in the articular lavage of 5-$LO^{-/-}$ mice and BOC2-treated WT mice. Some studies demonstrated that LXA_4 suppressed the production of IL-12 by dendritic cells *in vitro* and during infection with *Mycobacterium tuberculosis in vivo* (253,254). CD11c is an integrin expressed on macrophages/monocytes (255), non-conventional T cell subsets (256), NK cells (257) and mainly dendritic cells (258). We hypothesized that $CD11c^+$ cells in the joint could be dendritic cells. As a consequence, we checked for the presence of these cells in the lymph nodes. We detected increased numbers of activated $CD11c^+$ cells and $CD3^+CD4^+$ cells in 5-$LO^{-/-}$ mice. These results suggest that blockade of LXA_4 activity could increase the adaptive immune response against *S. aureus* infection. The migration of dendritic cells is mediated by the CCR7 receptor and its chemokine ligands CCL19 and CCL21 (259). Since we observed an increase in CD11c cells in lymph nodes of 5-$LO^{-/-}$ mice, we evaluated whether LXA_4 could have a role in the migration of dendritic cells through

CCR7. LXA_4 could decrease migration of human dendritic cells to CCL21 in a dose dependent manner. In order to demonstrate this role of LXA_4 in the dendritic cell migration, we need to perform more *in vitro* experiments such as chemotaxis assays with dendritic cells, CCL21 and BOC2 or WRW4. In addition, the expression of CXCR7 in dendritic cells *in vivo* during the S. aureus infection and during treatment with FPR2 antagonists needs to be evaluated.

Our hypothesis to explain the bacterial clearance in 5-$LO^{-/-}$ mice and in mice treated with FPR2 antagonists is that $CD11c^{+}$ cells can produce IL-12 and activated the Th1 response as demonstrated in infection with *Brucella abortus* (252) and *Mycobacterium tuberculosis* (249). We still have to measure these mediators in lymph nodes and joints. We also believe that these mediators can profoundly impact on bacterial killing, facilitating, for instance, a better activation of macrophages in the joint. Experiments to investigate the killing capacity of macrophages, ROS and nitric oxide production should be performed. We also checked the clinical relevance of LXA_4 blockade by using a combined treatment with BOC2 and vancomycin. Vancomycin is a glycopeptide antibiotic that acts through binding to peptidoglycan and via a complex alteration of cell wall synthesis. Since *S. aureus* presents resistance to methicillin, vancomycin has been largely used to treat *S. aureus* infection (260), including patients with septic arthritis. This combined therapy was more effective than BOC2 alone in this model. The single therapy with vancomycin was able to decrease the bacterial load and damage. A possible explanation for these results is the high concentration of antibiotic used in the treatment (400 mg/kg) and that the *S. aureus* strain used in this work is a very sensitive lab strain.

Taken together we showed that neutrophils are important for control of infection, articular damage and hypernociception in *S. aureus*-induced septic arthritis. The blockade of the chemokine system resulted in decreased neutrophil recruitment and the effect of this inhibition showed to be different depending on the approach used. The blockade of CXCR1/2 receptors in the late phase of disease was able to control the infection and decrease hypernociception. On the other hand, inhibition of GAG binding decreased neutrophil recruitment but was less effective in the control of infection and hypernociception. The blockade of 5-lipoxygenase, and particularly LXA_4, was able to decrease neutrophil recruitment, articular damage, hypernociception and bacterial load. LXA_4 negatively regulated the antigen presenting cell migration and adaptive immune activation, decreasing the ability of bacterial control.

CONCLUSION

Neutrophils have an important role in the bacterial control during *S. aureus*-induced arthritis, however are also related with articular damage and pain. The blockade of CXCR1/2, GAG binding and LXA_4 receptor was able to reduce part of these symptoms by decreasing neutrophil recruitment or improve adaptive immune response. These strategies can be useful as alternative therapies in the treatment of *S. aureus*-induced arthritis.

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SUPPLEMENTARY MATERIAL

SUPPLEMENT 1- ETHICS COMMITTEE CERTIFICATE

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo nº. 236 / 2012, relativo ao projeto intitulado "ESTUDO DOS MECANISMOS ASSOCIADOS COM A RESPOSTA INFLAMATÓRIA ARTICULAR EM UM MODELO DE ARTRITE SÉPTICA", que tem como responsável Flávio Almeida Amaral, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de 30/08/2012. Este certificado espira-se em 30/08/2017.

CERTIFICATE

We hereby certify that the Protocol nº. 236 / 2012, related to the Project entilted "STUDY OF THE MECHANISMS ASSOCIATED WITH THE INFLAMMATORY RESPONSE IN A MODEL OF SEPTIC ARTHRITIS", under the supervision of Flávio Almeida Amaral, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in 30/08/2012. This certificates expires in 30/08/2017.

FRANCISNETE GRACIANE ARAUJO MARTINS
Coordenador(a) da CEUA/UFMG

Belo Horizonte, 30/08/2012.

Atenciosamente.

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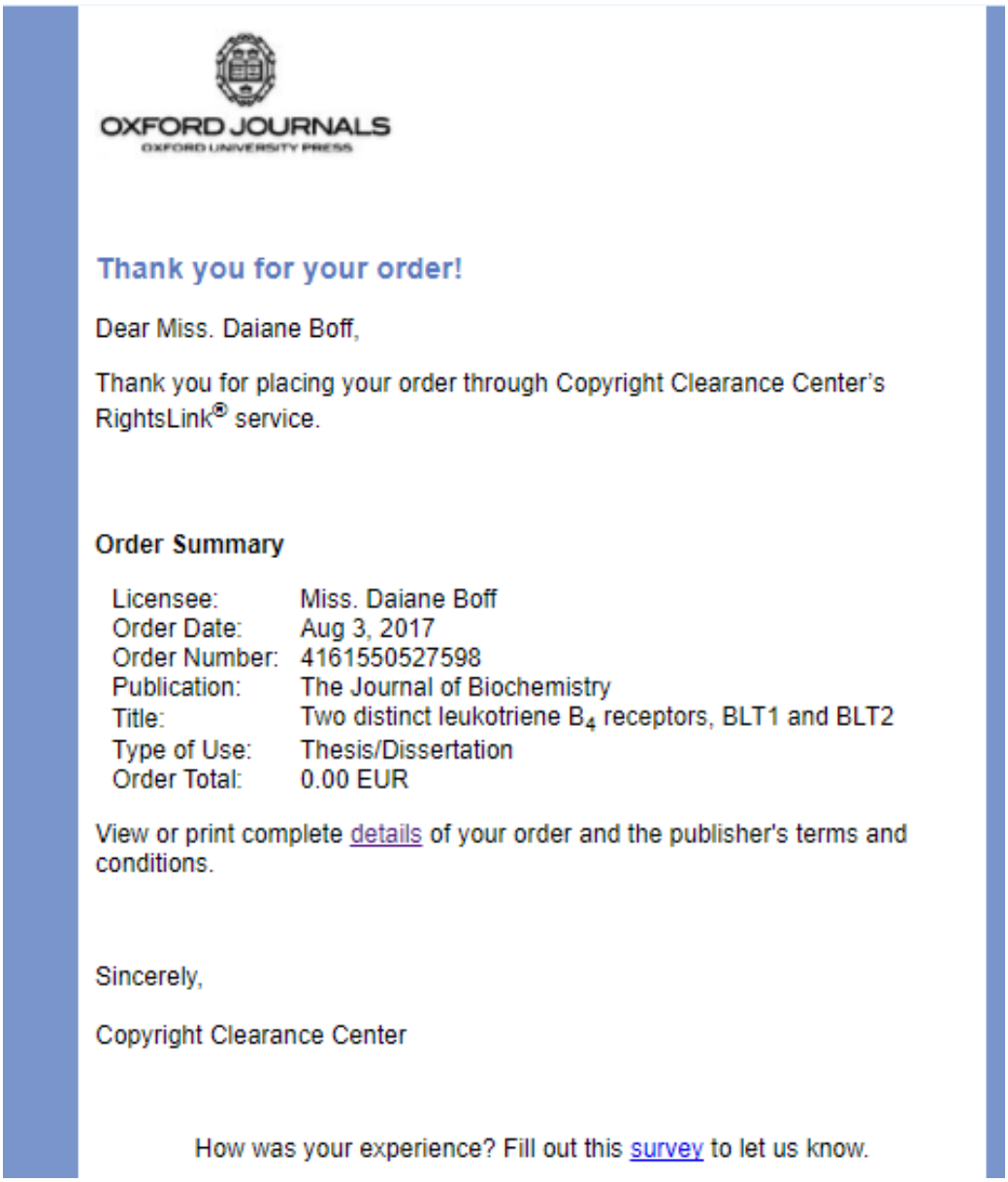

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SUPPLEMENT 3: ACCEPTED PAPER IN EUROPEAN JOURNAL OF IMMUNOLOGY

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

ABSTRACT

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

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 (Figure 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 (Figure 1A and B). The number of mononuclear cells increased later, peaking at day 14 and decreasing thereafter (Figure 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 (Figure 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 (Figure 2A and B). The first day of infection was characterized by a marked influx 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 (Figure 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 (Figure 2C). Joint dysfunction, as assessed by measuring hypernociception, was present throughout the observation period (Figure 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 (Figure 3A). Importantly, there was partial but not complete blockade of neutrophil influx into the joint (Figure 3B). Likewise the myeloperoxidase (MPO) activity (Figure 3C) was significantly reduced. There was no decrease in mononuclear cell numbers recovered from the joint cavity (Figure 3D). Production of the neutrophil attractant CXCL1 was significantly reduced in inflamed tissue (Figure 3E). In addition, treatment with DF2156A led to lower concentrations of TNF-α and IL-1β, comparable to levels found in uninfected mice (Figure 3F and G).

Treatment with DF2156A also led to reduced hypernociception, as evidenced by an increase of the withdrawal threshold in the flexed joint (Figure 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 (Figure 4B). In addition, this treatment efficiently reduced tissue (Figure 4C) and cartilage (Figure 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 Figure 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 (Figure 3B).

As shown in Figure 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 (Figure 5C and D). CXCL8 alone, without neutrophils, had no effect on the bacteria and DF2156A neutralized the effect of CXCL8 (Figure 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.

Delayed DF2156A treatment limits joint hypernociception and prevents the increase in bacterial load

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 Figure 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 (Figure 6A-C). Delayed treatment decreased joint pain early in the course of infection (day 4) but not at day 7 after infection (Figure 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: 1) 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; 2) 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; 3) CXCR2 activation is very important for the control of *S. aureus* infection by neutrophils into the joint; 4) 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 [42]. 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 efficacy to kill *Pseudomonas aeruginosa* [40] and *Candida albicans* [41] 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 [42]. Once activated, neutrophils secrete granules, enzymes, reactive oxygen species and some antimicrobial peptides that can damage the tissue [43],[44]. 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 [45],[46]. In this context, the absence of IL-1 and its receptors impairs bacterial elimination in septic arthritis [47]. However, the presence of *S. aureus per se* can produce and release bacterial enzymes and virulence factors that directly lead to tissue damage [48],[49]. 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 [50]. 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 [51]. 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 [52],[53]. On the

other hand, CXCR1/2 and their ligands also contribute to pain by direct activation of afferent nociceptive fibers [54]. Interestingly, a study showed that *S. aureus* can directly trigger action potentials in nociceptive neurons through N-formylated peptides and α-haemolysin toxin [55]. 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 to 50% of patients have permanent dysfunction of the affected joint [56]. 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 [57],[58]. 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 [59],[60]. Experimentally, the combined therapy of antibiotics with anti-TNF [61] or corticosteroids and bisphosphonate [62] 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 37ºC 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 [63]. 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 hours. 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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TABLES

Table 1

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.

FIGURES

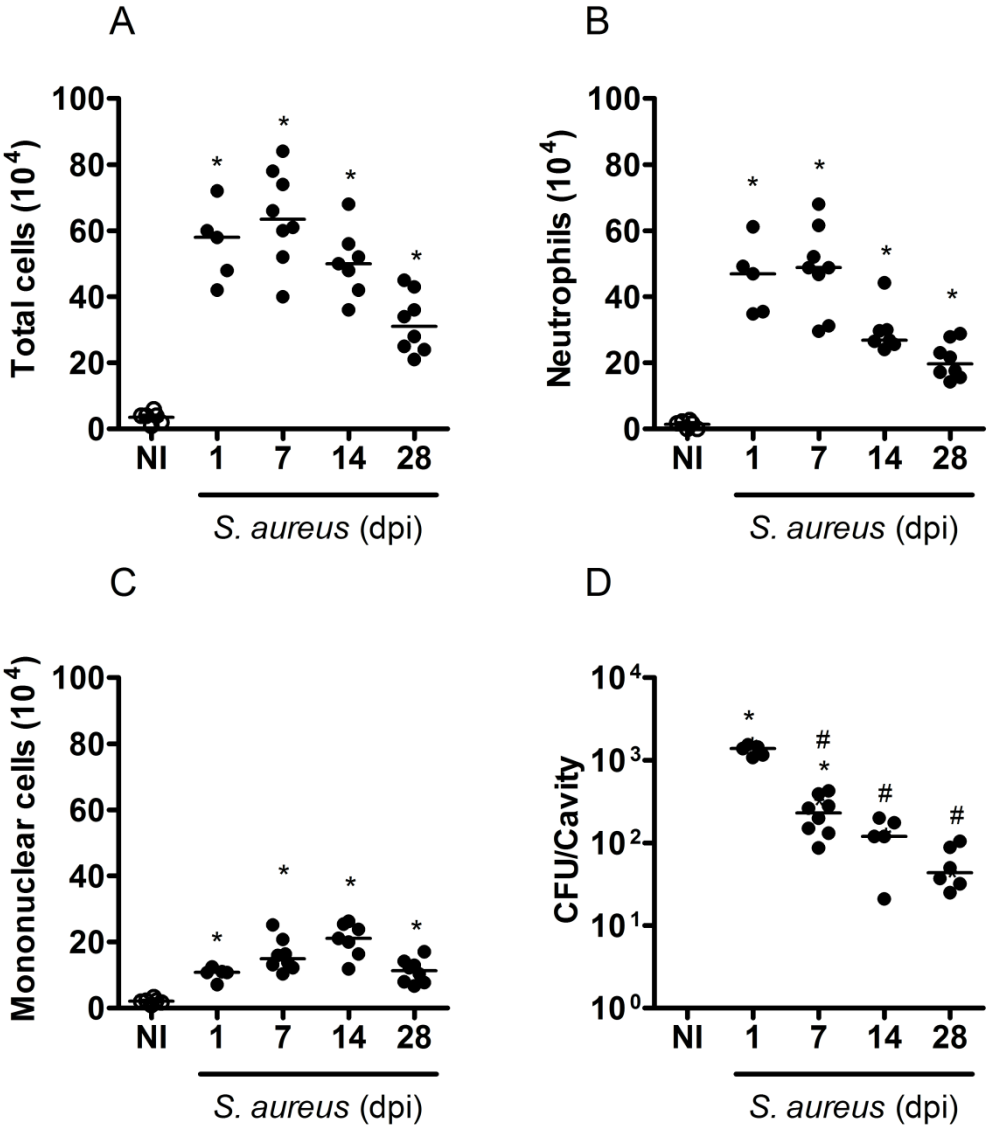

Figure 1: Kinetics of cell recruitment and bacterial load in the joint. Mice were injected intra-articularly with *S. aureus*. Cells were harvested from the cavity 1, 7, 14 or 28 days after injection. (A) The total number of leukocytes, (B) neutrophils and (C) mononuclear cells recruited to the joint 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.

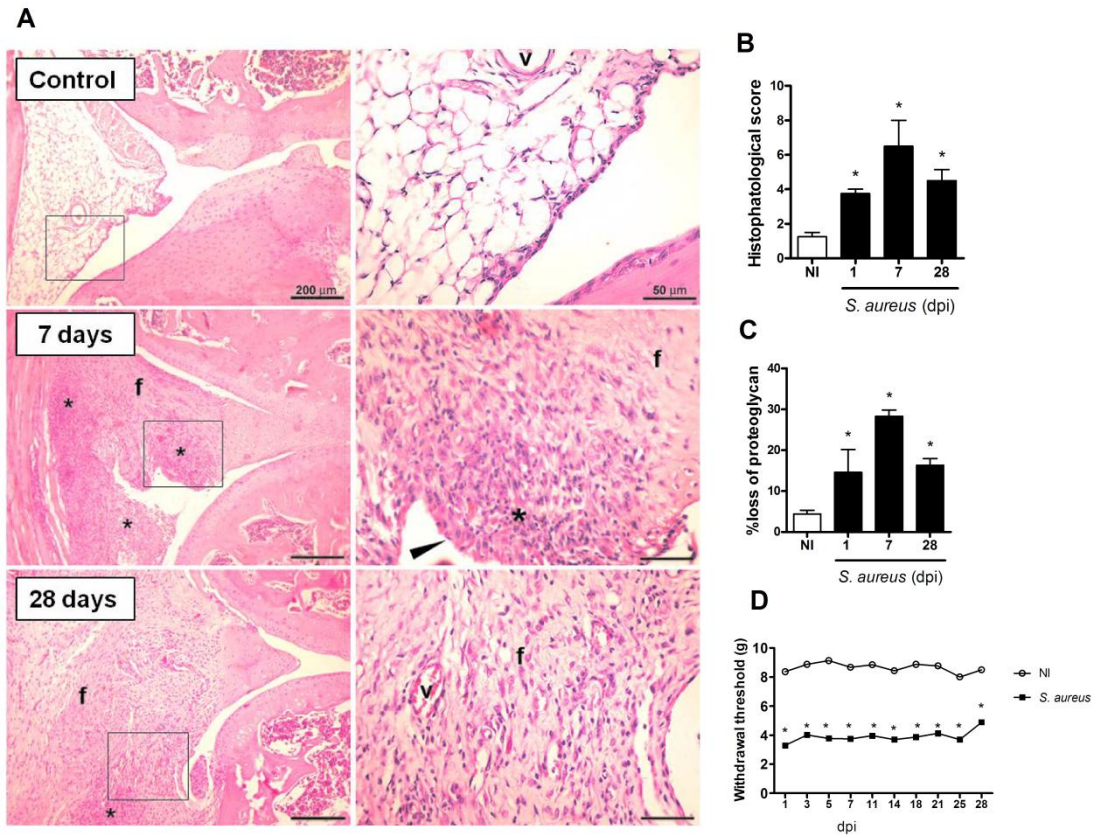

Figure 2: Kinetics of articular damage and hypernociception in *S. aureus*-induced arthritis. The joint was removed 1, 7 or 28 days after *S. aureus* infection and tissues processed for histopathological analyses. (A) Representative images of joints (v - blood vessels; f - collagen fibers; * - cellular 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.

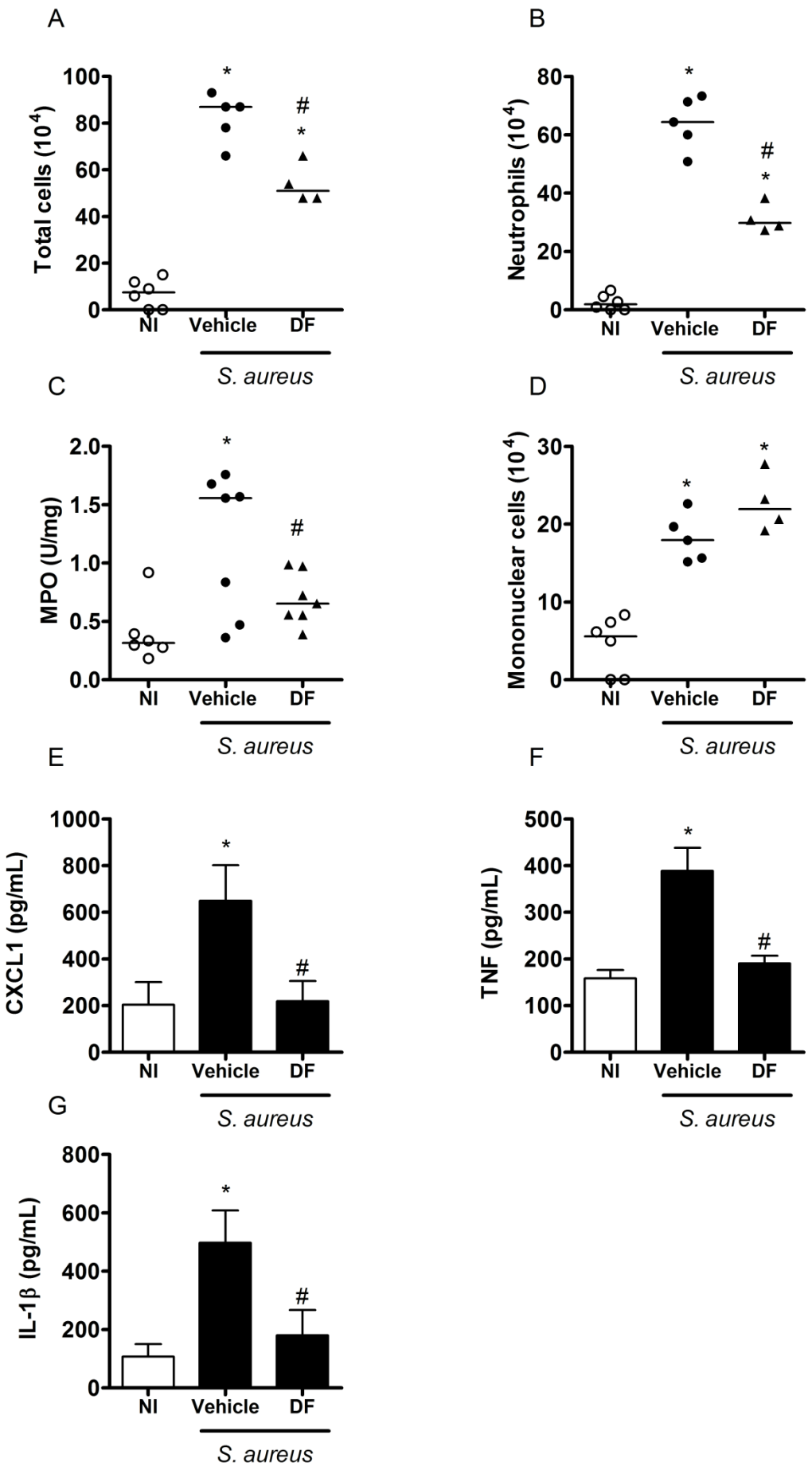
A
Total cells (10^4)
100
80
60
40
20
0
NI
Vehicle
DF
S. aureus
B
Neutrophils (10^4)
80
60
40
20
0
C
MPO (U/mg)
2.0
1.5
1.0
0.5
0.0
D
Mononuclear cells (10^4)
30
20
10
0
E
CXCL1 (pg/mL)
1000
800
600
400
200
0
F
TNF (pg/mL)
500
400
300
200
100
0
G
IL-1β (pg/mL)
800
600
400
200
0

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 hour prior 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.

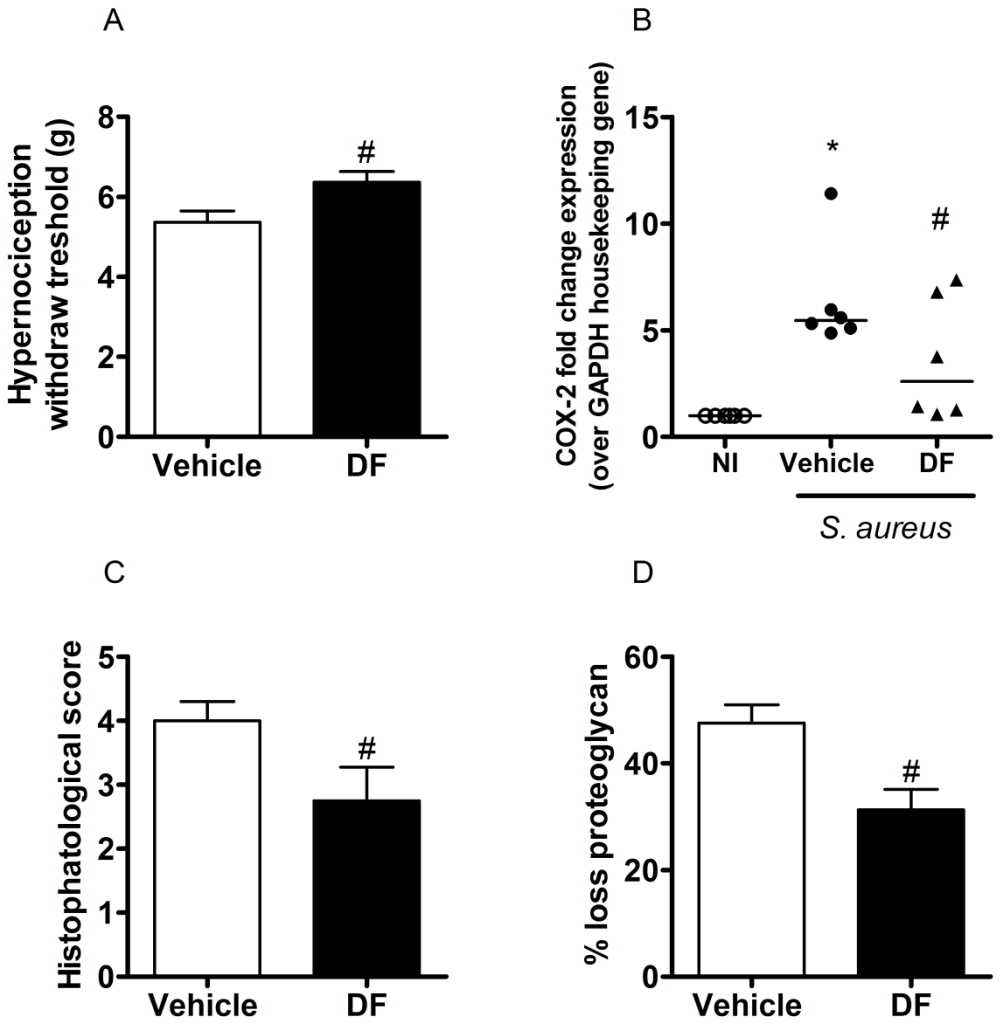

Figure 4: The blockade of CXCR1/2 decreased hypernociception and tissue damage in *S. aureus*-infected joint. Mice were infected with *S. aureus* into the tibiofemoral joint and evaluated 7 days later. A group of mice was treated with DF2156A 1 hour prior the injection of *S. aureus* and daily for the 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.

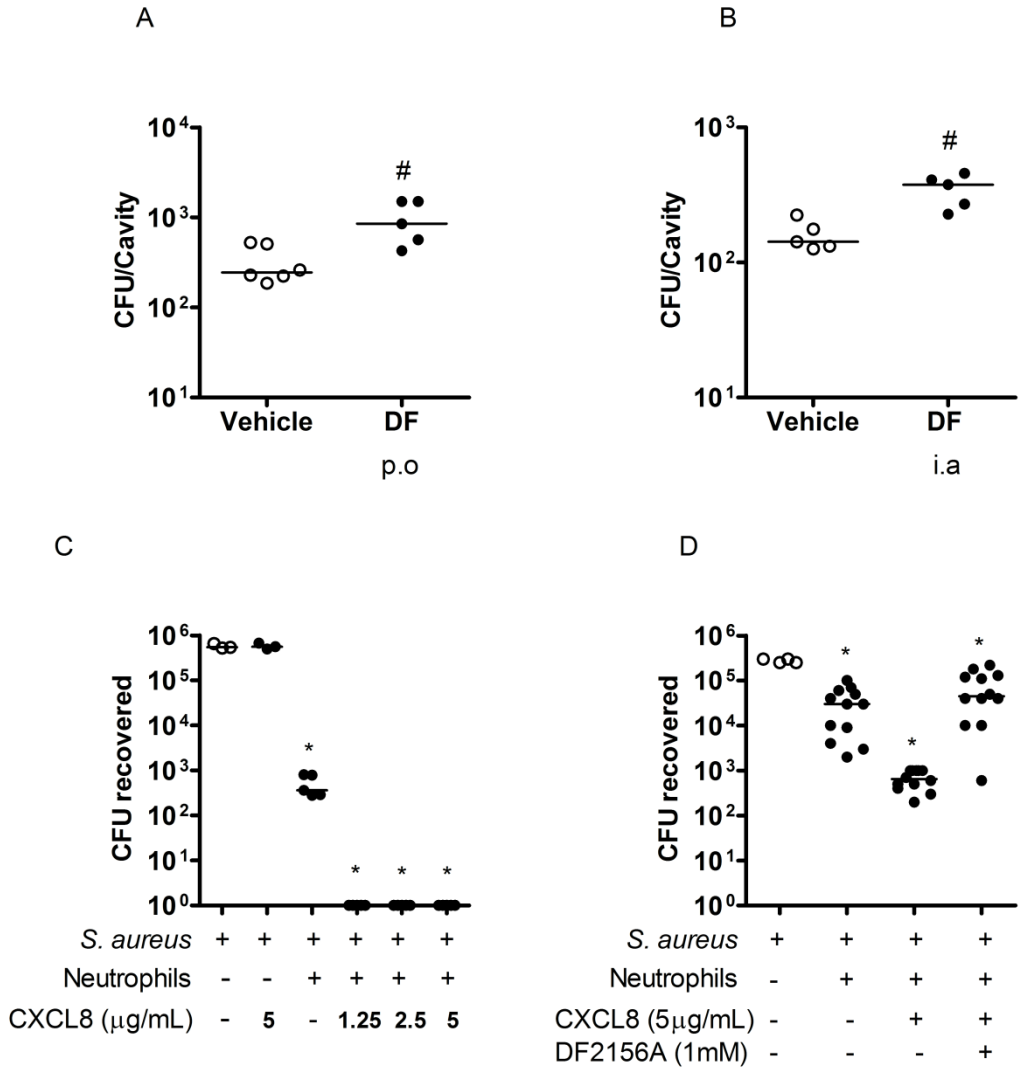

Figure 5: CXCR1/2 are important for the control of *S. aureus*. Mice were infected with *S. aureus* into the tibiofemoral joint and evaluated 7 days later. The inflamed periarticular tissue was collected for the analysis of the bacterial load. (A) A group of mice were treated with DF2156A 1 hour prior 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 hours 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.

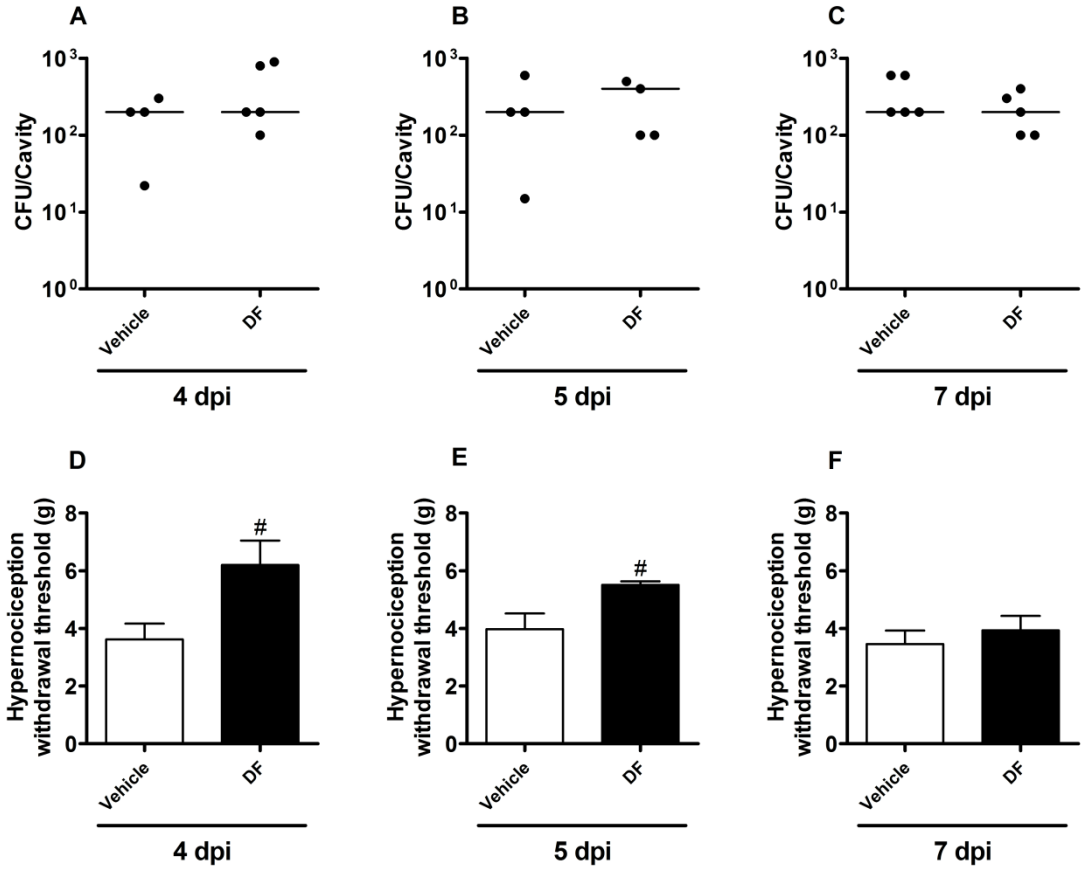

Figure 6: The blockage of CXCR1/2 in a late stage decreases hypernociception and prevents the increase in bacterial load. Mice were infected with *S. aureus* into the tibiofemoral joint and treatment was started 3 days after infection (dpi). Different groups of mice were treated daily with DF2156A or 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.