UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Bioquímica e Imunologia

Rodrigo Uribe Alvarez

BLOCKING CXCR1/2 CHANGES NEUTROPHIL MHCII SURFACE EXPRESSION AND DECREASES INFLAMMATION IN A MURINE MODEL OF PROLONGED ARTHRITIS

Belo Horizonte
2019

Rodrigo Uribe Alvarez

BLOCKING CXCR1/2 CHANGES NEUTROPHIL MHCII SURFACE EXPRESSION AND DECREASES INFLAMMATION IN A MURINE MODEL OF PROLONGED ARTHRITIS

Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Imunologia da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Bioquímica e Imunologia.

Orientador: Prof. Dr. Mauro Martins Teixeira

Co-orientador: Prof. Dr. Flávio Almeida Amaral

Belo Horizonte

2019

043 Alvarez, Rodrigo Uribe.
Blocking CXCR1/2 changes neutrophil MHCII surface expression and decreases inflammation in a murine model of prolonged arthritis [manuscrito] / Rodrigo Uribe Alvarez. – 2019.

73 f. : il. ; 29,5 cm.

Orientador: Dr. Mauro Martins Teixeira. Coorientador: Dr. Flávio Almeida Amaral.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica e Imunologia.

1. Artrite. 2. Receptores de Interleucina-8A. 3. Receptores de Interleucina-8B. 4. Antígeno B7-2. 5. Neutrófilos. 6. Inflamação. I. Teixeira, Mauro Martins. II. Amaral, Flávio Almeida. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 577.1

Ficha catalográfica elaborada pela bibliotecária Rosilene Moreira Coelho de Sá – CRB 6 – 2726

Universidade Federal de Minas Gerais
Curso de Pós-Graduação em Bioquímica e Imunologia ICB/UFMG
Av. Antônio Carlos, 6627 – Pampulha
31270-901 – Belo Horizonte – MG
e-mail: pg-biq@icb.ufmg.br (31)3409-2615

ATA DA DEFESA DA TESE DE DOUTORADO DE RODRIGO URIBE ALVAREZ. Aos sete dias do mês de junho de 2019 às 14:00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado ""Avaliação do efeito de um antagonista de CXCR2 na resposta inflamatória em um modelo de Artrite induzida por antígeno em camundongo"", requisito final para a obtenção do grau de Doutor em Ciências: Imunologia. Abrindo a sessão, o *Presidente da Comissão, Prof. Mauro Martins Teixeira, da Universidade Federal de Minas* Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dr. Luíz Carlos Crocco Afonso (Universidade Federal de Ouro Preto), aprovado; Dr. Francisco Airton Castro da Rocha (Universidade Federal do Ceará), aprovado; Dra. Vanessa Pinho (Universidade Federal de Minas Gerais), aprovado; Dr. Luis Henrique Franco (Universidade Federal de Minas Gerais), aprovado; Dr. Flávio Almeida Amaral - Coorientador (Universidade Federal de Minas Gerais), aprovado; Dr. Mauro Martins Teixeira - Orientador (Universidade Federal de Minas Gerais), aprovado. Pelas indicações o candidato foi considerado:

(X) APROVADO
( ) REPROVADO

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 07 de junho de 2019.

Dr. Luíz Carlos Crocco Afonso (Universidade Federal de Ouro Preto)

Dr. Francisco Airton Castro da Rocha (Universidade Federal do Ceará)

Dra. Vanessa Pinho (UFMG)

Dr. Luis Henrique Franco (UFMG)

Dr. Flávio Almeida Amaral - Coorientador (UFMG)

Dr. Mauro Martins Teixeira - Orientador (UFMG)

Profª Lêda Quércia Vieira
Coordenadora do Curso de Pós Graduação

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Professor Mauro Martins Teixeira and Professor Flávio Almeida Amaral, my research supervisors, for their guidance, encouragement, useful critiques and constructive suggestions during the planning and development of this research work. Their willingness to give their time so generously has been very much appreciated.

My sincere thanks also goes to the professors that accepted the invitation to be part of my thesis evaluation committee.

I would like to thank CAPES, CNPq and FAPEMIG for their financial support throughout the development of this work.

I would like to thank the Biochemistry and Immunology department of the UFMG.

I would also like to extend my thanks to the technicians of the laboratory for their help throughout the development of this research work. My grateful thanks are also extended to my fellow students and group professors who somehow contributed to the development of this work.

Finally, I wish to thank my family for their support and encouragement throughout my studies.

RESUMO

A artrite reumatoide (AR) é uma doença crônica e autoimune que afeta as articulações, músculos e tendões, causando dor intensa e deformidade articulares. Afeta aproximadamente 1% da população mundial, majoritariamente mulheres acima de 35 anos de idade. O desenvolvimento da AR está associado com fatores ambientais e genéticos que levam ao paciente a perder tolerância imunogênica. A inflamação e o dano das articulações na AR são consequências de um infiltrado celular intenso no tecido e fluido sinoviais, grande parte constituído por neutrófilos que são recrutados por diferentes moléculas quimioatraentes. Os receptores de quimiocinas CXCR1/2 são expressos na membrana plasmática de neutrófilos e podem se ligar a alguns ligantes da quimiocinas da família CXC. Quando os neutrófilos são ativados e recrutados para os tecidos, são capazes de secretar grânulos e citocinas pró-inflamatórias. Além de serem células efetoras da resposta imune inata, os neutrófilos parecem exercer funções características de resposta imune adaptativa. Neste trabalho, investigamos se o bloqueio dos receptores CXCR1/2 reduz as funções inatas e adaptativas dos neutrófilos na artrite-induzida por antígeno (AIA) em camundongos. Cinco dias após o desenvolvimento da artrite, numa fase de intensa resposta inflamatória, neutrófilos presentes na cavidade articular, linfonodo drenante e baço expressavam moléculas características de células apresentadoras de antígenos, como MHCII e CD86. Animais tratados com um antagonista de CXCR1/2, Reparixina, tiveram redução no número de neutrófilos acumulados dentro da cavidade articular, menor dano tecidual e reposta hipernociceptiva quando comparados com animais artríticos sem tratamento. De maneira interessante, animais artríticos tratados com Reparixina a partir do 5º dia da artrite tinham menor expressão de MHCII nos neutrófilos. O bloqueio de CXCR1/2 também diminui a ativação de linfócitos, a produção de IFNγ *in vivo* e a produção de IL-17 de esplenócitos reestimulados *ex vivo*. Portanto, os receptores CXCR1/2 parecem ter diferentes efeitos na patogênese da inflamação articular, atuando nas respostas imunes inata e adaptativa, contribuindo direta ou indiretamente até mesmo na sensibilização dos neurônios no modelo de artrite usado neste estudo. Assim, os

receptores CXCR1/2 podem ser alvos terapêuticos importantes para o controle da inflamação em pacientes com doenças artríticas inflamatórias como a AR.

Palavras chave: Artrite. Neutrófilos. Inflamação. CXCR1. CXCR2. Reparixina. MCHII.

ABSTRACT

Rheumatoid Arthritis (RA) is an autoimmune, inflammatory, and chronic disease that causes pain, harm in the joints, loss of cartilage and bone destruction. RA is associated with multifactorial agents (genetic and environmental) that lead to loss of self-tolerance. There is no cure for it and in consequence, it still affects nearly 1% of the world's population, mostly women over 35 years old. Pain and tissue injury in RA are associated to a large number of leukocytes infiltrated into the inflamed synovial cavity and membrane, and neutrophils are active cells in its pathogenesis. The migration of neutrophils from bloodstream to tissue is guided by several chemoattractant molecules, including chemokines. CXCR1/2 chemokine receptors are expressed on neutrophil surface and bind to some CXC ligands. Once activated and into the tissues, neutrophils secrete granules containing numerous toxic compounds and pro-inflammatory cytokines. Neutrophils were once described as innate immunity effector cells with no relevant purpose in the adaptive immunity phase. However, in the last couple of decades, neutrophils have emerged as cells whose role extends into adaptive immunity. Here, we aimed to investigate if the blockade of CXCR1/2 could reduce innate and adaptive functions of neutrophils during the pathogenesis of prolonged antigen-induced arthritis (AIA) in mice. Neutrophils retrieved from the synovial cavity, the draining popliteal lymph node and the spleen of AIA mice, presented increased MHCII and CD86 expression, two key markers of antigen presenting cells for T cell activation. The systemic blockade of the CXCR1/2 receptors with Reparixin in the peak of the inflammatory response decreased the quantity of neutrophils recruited and their MHCII expression, leading to the reduction of joint tissue damage and hypernociception in AIA mice. Blockade of CXCR1/2 decreased lymphocyte activation, as seen by decreased production of IFN-γ in vivo and IL-17 production by splenocytes. Thus, CXCR1/2 could have multifactorial effects on joint pathology, ranging from innate to adaptive immune response and contribution to neuron sensitization in the model of arthritis used in this study.

Keywords: Arthritis. Neutrophils. Inflammation. CXCR1. CXCR2. Reparixin. MHCII.

LIST OF FIGURES AND TABLES

ABBREVIATIONS

AIA	Antigen induced arthritis
ACPA	Anti-citrullinated protein antibodies
APC	Antigen presenting cell
BSA	Bovine serum albumin
CXCR1/2	Chemokine receptors CXC1 and CXC2
ELISA	Enzyme-Linked Immunosorbent Assay
HLA-DR	Human leukocyte antigen-DR isotype
FACS	Fluorescence-activated cell sorting
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
FSC vs SSC	Forward *versus* side scatter
H&E	Hematoxylin and eosin
IFNγ	Interferon gamma
LN	Lymph node
LTB4	Leukotriene B4
mBSA	Methylated bovine serum albumin
MHCII	Major histocompatibility complex II
MLS	Macrophage-like synoviocytes
MFI	Mean fluorescence intensity
MPO	Myeloperoxidase
NK	Natural killer
NETs	Neutrophil Extracellular Traps
PCh	Post challenge
PMN	Polymorphonuclear
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
TNF-α	Tumoral necrosis factor α
T1 and T2	Treatment 1 and Treatment 2

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Rheumatoid Arthritis

Inflammation is a host´s response to infection or tissue injury whose purpose is to eliminate the causing noxious factor, restore tissue homeostasis and in the case of infections, promote immunologic memory that will help the host react faster against a reinfection by the same infectious agent [1-4]. However if inflammation is not controlled and/or fails to resolve, as in persistent injuries, long-term infections, autoimmune responses, or prolonged exposures to toxic agents, it can give rise to chronic inflammation, which leads to tissue deterioration and disease [3, 5].

Rheumatoid arthritis (RA) is an autoimmune, inflammatory, chronic disease, which causes harm in the joints, loss of cartilage and bone destruction affecting nearly 1% of the world population [6-8]. Pain and tissue injury in RA are mainly due to the large amount of leukocytes infiltrated into the inflamed synovial cavities, were neutrophils are the main cell recruited [6, 9].

Genetic factors influence RA incidence as monozygotic twins have a 12-15% chance of having RA, compared to 1% of the general population [10]. Furthermore, epigenetic factors like smoking and periodontal disease are also involved in the development of RA [11, 12]. There are more than 100 loci associated with RA, the majority involved in immune mechanisms and therefore could be used as therapeutic targets [13].

The pathogenesis of RA is associated with some variations in the Human leukocyte antigen-DR isotype (HLA-DR) genes, which are surface receptors for MHC class II [14]. Some genotypes, like HLA-DRB1, are related to a more erosive and aggressive disease that results in an increase in mortality rates [14].

Several autoantibodies like rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) can be detected in the serum of RA patients; hence they are used as markers for RA diagnosis [15, 16]. The formation of RF and ACPA is associated

with genetic factors, like HLA alleles and environmental factors like smoking [15]. ACPA can predict disease severity since their presence is linked to bone erosion and pain in RA patients [15, 16].

RA symptoms are consequence of the activation state and the large quantities of leukocytes infiltrated in the inflamed synovial cavities. Among this leukocytes are granulocytes, macrophages, lymphocytes T $CD4^+$, T $CD8^+$ and B cells [6]. These leukocytes secrete cytokines like TNF-α, IL-17, IL-6 e IL-1β, chemokines and different proteases that are the main contributors to the progression of RA [6, 17].

The synovium is a soft tissue that forms a membrane at the edge of the joints and provides lubrication and nutrients for the cartilage. It has an outer layer and an inner layer formed of a mixture of macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS). This layers are affected in RA, were inflammation transforms this healthy lining structure into a pannus-like structure. This structure extends into the joint space forming a pannus-cartilage junction were joint destruction occurs through the production of cytokines, chemokines and by cell recruitment towards the joint cartilage [18].

Nevertheless, if RA is diagnosed early, the initial treatment can control inflammation and help prevent subsequent damage. The blockade of several inflammatory mediators secreted by infiltrated leukocytes along with the inhibition of cellular recruitment and activation of these leukocytes has had beneficial results with RA patients. These therapies include non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti rheumatic drugs (DMARDs), glucocorticoids, synthetic receptor agonists or antibodies that inhibit certain proinflammatory cytokines [19]. However, this treatments can be expensive, some patients do not respond well to the treatments, and they can let the patient in a immunosuppressed state [20]. Therefore, understanding better the mechanisms of this disease, through clinical and translational studies, is fundamental to propose new therapeutic options.

In order to study RA, the development of several animal models has been essential. Each one has certain characteristics that reproduce aspects of RA in humans, and although all of them have shortcomings, they help in the understanding

of the pathogenesis of RA and the screening of potential drugs and their targets. The most common animal models include antigen-induced arthritis (AIA), collagen-induced arthritis (CIA), tumor necrosis factor-α (TNF α) transgenic mouse model, proteoglycan-induced arthritis (PGIA), streptococcal cell wall (SCW)–induced arthritis and K/BxN-transgenic model. All of them have specific characteristics used to study specific components and stages of the inflammatory process in RA [21].

Murine models of antigen-induced arthritis (AIA) can be helpful tools for the study of the cellular and molecular mechanisms of RA, because they have histopathological and immunological resemblance with RA [22, 23]. The mice model of AIA induced with methylated bovine serum albumin (mBSA) reproduces some characteristic parameters of RA, like cytokine and chemokine production, tissue damage, pain and similar cellular recruitment, confirmed by various studies [22, 24-26]. Among these recruited cells, T cells have an important role in the pathogenesis of AIA, as blocking CD4 has had a strong effect on macrophages, diminished inflammation and reduced joint swelling and destruction [27]. However, neutrophils are the main cells recruited towards the inflammation site in AIA and therefore are crucial in its pathogenesis [28, 29]. In the knee joints of AIA mice and RA patients, high levels of CXCL1, CXCL5 and leukotriene B4 (LTB4) have been expressed and when these chemokines have been blocked in AIA mice, there has been a reduction of neutrophil migration into the challenged knee joint [28]. Furthermore, blocking the CXCR2 receptor in AIA mice decreased neutrophil recruitment through inhibition of neutrophil adhesion to synovial micro vessels, and led to decreased cytokine production, hypernociception and tissue damage [30].

To study the role of neutrophils in the context of chronic inflammation, we used a prolonged inflammatory Antigen-induced arthritis (AIA) mice model. In most AIA models inflammation is acute, however the prolonged inflammatory model allows for a better differentiation of the stages of the inflammatory process [29, 31]. It is important to acknowledge that there are no animal models that mimic all the events that occur in Rheumatoid Arthritis in humans. However, a specific question about the

mechanism of action of a molecule or cell will favor a certain experimental model [22, 23].

The prolonged inflammatory AIA model is induced by intradermal immunization with mBSA and direct challenge with mBSA into the knee joint cavity [22, 24-26]. As in different models of arthritis, depletion of neutrophils and treatment with compounds that block neutrophil recruitment in the AIA model have caused reduction in the severity of the inflammatory response [32-36]. Therefore, the role of neutrophils in the model of prolonged AIA became the main focus of this study.

1.2 Neutrophils

Neutrophils are the first cells recruited from the bone marrow to the infection or damage site, where they are activated through direct contact with the pathogen or damaged tissue, or throughout interaction with proinflammatory cytokines secreted by resident cells [37, 38]. Once activated, they secrete granules containing numerous toxic compounds as reactive oxygen species (ROS) and proteases in order to eliminate the causing agent of inflammation. This potent effector molecules cannot single out the infectious or noxious agent from the hosts self and therefore subsequent tissue damage is inevitable (Figure 1) [37].

Another mechanism of pathogen clearing from neutrophils is the release of Neutrophil Extracellular Traps (NETs), which are made out of cytoplasmic proteins, extracellular chromatin and immune response mediators (Figure 1) [39]. Although the main function of NETs is to capture pathogens to avoid their dissemination, their accumulation can be linked to adverse consequences, like epithelial and endothelial damage [39, 40]. NETs are comprised of large quantities of histones, which are targets of citrullination and therefore, become new antigens for anti-citrullinated protein autoantibodies (ACPA) to recognize in RA patients [41]. NETs can also stimulate proinflammatory responses in synovial fibroblasts of patients with RA, including the production cytokines, chemokines, and adhesion molecules [42]. However, NETs can promote the resolution of inflammation through the degradation of cytokines and chemokines and disrupting neutrophil recruitment and activation by the activation of serine proteases [43].

Neutrophils are involved in joint tissue destruction in RA. Immune complexes that can be found in the synovial fluid and on the articular cartilage in patients with RA, can activate these neutrophils, which would trigger the release of ROS [44]. The level of ROS in neutrophils from patients with RA is higher than from neutrophils from healthy individuals, and treatment with antioxidants has diminished joint destruction and therefore decreased the arthritis index in murine models of arthritis [45-47]. The infiltration of neutrophils is directly associated with tissue damage, pain and

aggravation of the inflammatory clinical condition [9]. Thus, compounds that target neutrophils can be useful to treat numerous inflammatory diseases.

Neutrophils were once described as innate immunity, effector cells, with no relevant purpose in the adaptive immunity phase. However, neutrophils have been proven to be cells whose role not only can extend into the adaptive immunity but that can also act as a bridge between the innate and the adaptive immunity [48]. Neutrophils can increase their lifetime during the inflammatory process and acquire phenotypic and functional properties that are typically reserved for professional antigen presenting cells (APCs) [48].

APCs are immune cells that process protein antigen, breaking it into peptides and present it on the cell surface in conjunction with class II major histocompatibility complex (MHC) molecules for interaction with appropriate T cell receptors [49]. Classical APCs include dendritic cells, macrophages, Langerhans cells and B cells, and they act as a link between the innate and adaptive immune responses. In order to activate antigen-specific T cells, APCs display antigen-MHCII on the membrane together with co-stimulatory signals like CD86 and CD80 [49].

Neutrophils obtained from the synovial cavity of RA patients transcribe and express major histocompatibility complex type II (MHCII), unlike neutrophils obtained from healthy individuals [50]. Although these neutrophils expressed low levels of the costimulatory molecules CD86 and CD80, they were still able to stimulate T cell proliferation [50].

Neutrophils obtained from the inflamed colon of mice subjected to a T cell transfer model of chronic colitis had an enhanced MHCII and CD86 surface expression when compared to blood and spleen neutrophils [51]. Even more, these extravased neutrophils acquired APC-like functions in the site of inflammation, which rendered them able to induce proliferation of ovalbumin-specific $CD4^+$ T cells in an MHCII-dependent manner. In consequence, neutrophils were able to perpetuate chronic inflammation by inducing T cell activation and proliferation as well as by enhancing production of pro-inflammatory mediators [51].

Neutrophils have been able to present antigen to autologous antigen-specific $CD4^+$ T cells in a MHC-II-dependent manner in-vitro [52, 53]. Additionally, neutrophils were able to prime Th1 and Th17 differentiation of T cells in-vitro [53]. Furthermore, neutrophils sorted from vaccine-draining lymph nodes from rhesus macaques could present vaccine antigen to autologous antigen-specific memory $CD4^+$ T cells ex-vivo [52]. Neutrophils could also help in the elimination of viral infections by acting as APCs for TCD8+ cells, as shown in lungs infected with influenza virus [54]. Neutrophils can also express CCR7, a chemokine receptor that causes neutrophils to migrate through the lymph vessel towards the lymph nodes, were T cells reside, which would allow for neutrophils to move as APCs and to be able to modulate the adaptive immune response[55].

Neutrophils can also influence the immune response by doing cross-talk with different cells throughout different mechanisms and therefore have a role in orchestrating the immune response [56]. When neutrophils are activated by TLR agonists, they are able to do cross-talk with Natural killer (NK) cells though the release of the soluble mediators IL-8, MIP-1α, and MIP-1β in-vitro [57]. The activation of NK cells by neutrophils makes them induce monocyte-derived dendritic cell maturation and to promote activation and clonal expansion of T cells and IFN-γ production, which could drive an adaptive immune response [57]. Furthermore, neutrophils can induce the migration of T cells towards inflammation sites. In a model of influenza in mice, TCD8+ cells were guided towards the infection site by CXCL12-containing trails liberated by neutrophils during their migration [58]. Moreover, the absence of these neutrophils impaired the establishment of a sustained $CD8^+$ T cell population at the site of infection through altered $CD8^+$ T cell traffic and localization [58].

Neutrophils die via apoptosis following the activation and uptake of pathogens or at the end of their lifespan. Apoptotic neutrophils are phagocytized by macrophages, causing an alteration in the functionality of these macrophages by becoming cells with an anti-inflammatory profile [59]. Apoptosis is essential for the removal of neutrophils from inflamed tissues and for the timely resolution of

neutrophilic inflammation. When neutrophilic apoptosis has been induced in a model of AIA, inflammation and tissue damage has decreased [60]. Other pro-resolutive functions from neutrophils comprise their capacity to secrete Annexin A1, which recruits monocytes and causes apoptosis in neutrophils contributing to the process of resolution of inflammation [61]. Apoptotic neutrophils can also maintain their receptors for pro inflammatory cytokines to act as a decoy. These proinflammatory cytokines could bind the receptors from the apoptotic cells instead of receptors from viable cells, and therefore they would not be able to signalize [62].

In different arthritis models in animals, the depletion of neutrophils has had a reduction in the severity of the inflammatory response [32, 33]. Also, the treatments with compounds that block neutrophil recruitment are able to reduce the inflammatory response and the hypernociceptive response [34-36].

This makes neutrophils the target of studies to better understand the pathogenesis of diverse diseases and to be able to optimize future treatments [63]. Although current anti-inflammatory therapies focus on the inhibition of leukocyte recruitment and decreasing the amount of pro-inflammatory mediators, there is a great interest for the strategies which target the resolution of the inflammatory response [19]. Among these strategies can be cited the changing of phenotype of proinflammatory cells to pro-resolutive phenotypes, mainly targeting polymorphonuclear cells and apoptosis [64, 65].

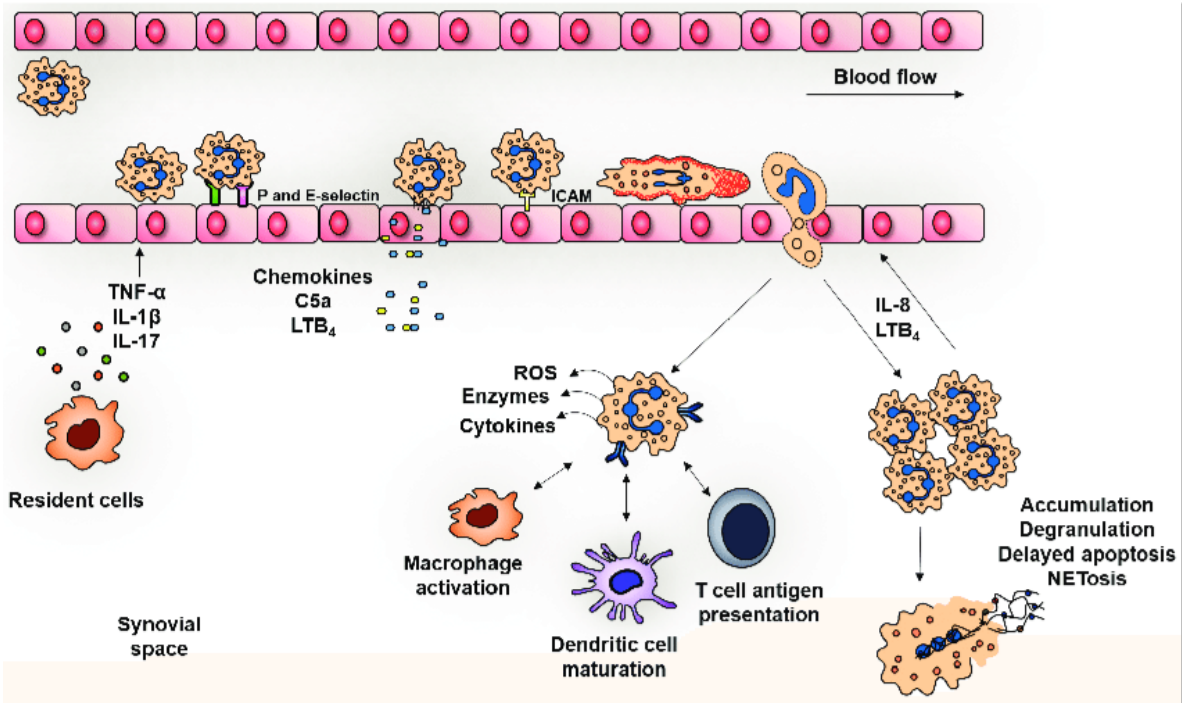

Figure 1. Neutrophil recruitment.
Figure adapted from Rosas et al. 2017 [66]. Neutrophils are recruited by chemotactic signals like TNF-α, IL1β, C5a and LTB4. Neutrophils extravasation towards the injured or infected tissue occurs as a result of the activity of adhesion molecules. Neutrophils are activated by other immune cells or by molecules, causing to liberate ROS and other enzymes that contribute to the pathogenesis of inflammatory arthritis diseases. Once activated, neutrophils liberate cytokines and chemokines, or interact directly with other inflammatory cells to boost the inflammatory response. Neutrophils are also capable of making NETs that contribute to tissue damage. The cytokines produced by activated neutrophils can recruit more neutrophils and therefore causing a prolonged inflammation and subsequent tissue damage. Therefore an effective apoptosis is required to achieve the resolution of inflammation.

1.3 CXCR1/2 receptors and their ligands

Chemokines are small peptides (~7-15kDa) that stimulate cellular migration, mainly leukocytes, through interactions with G protein coupled receptors which share a common structure characterized by 7 transmembrane domains [67]. The signaling pathways controlled by chemokines are induced by their binding to the G protein coupled receptors, resulting in the recruitment and activation of leukocytes in inflammatory or physiological situations [68]. Besides G protein coupled receptors the chemokine system also includes atypical chemokine receptors (ACKRs), which are known as decoy receptors. When ACKRs bind chemokines, they internalize them and degrade them, acting as negative regulators of the inflammatory response [69, 70]. Structurally, both chemokine receptor groups distinguish themselves because of the DRYLAIV aminoacid sequence, present in the end of the third transmembrane domain of G protein coupled receptors, but absent in the atypical receptors [71].

Chemokines share a common topology consisting in a N-terminal portion, conserved cysteine motifs, a N-loop and a C-terminal portion. Chemokines are classified in 4 big families depending on the position of the last 2 cysteines that are closer to the N-terminal portion: CC, CXC, CX3C and XC [72, 73]. In chemokines of the CC family, the 2 molecules of cysteine are together, whilst the chemokines of the CXC family have an amino acid between them. Chemokines of the CX3C family have 3 amino acids between the 2 cysteines while the chemokines of the XC family lack the 1st and 3rd cysteine of the motif. To name one determinant ligand and their receptor, the nomenclature used is CC, CXC, XC or CX3C followed by the L (for ligand) or the R (for receptor) and a number. Examples of this could be CXCL1 and CXCR1 [73].

CXC chemokines that have N-terminal glutamic acid–leucine–arginine (ELR) sequence immediately preceding the CXC motif (CXCL ELR$^+$ chemokines) are potent neutrophil chemoattractants and comprise CXCL1, 2, 3, 5, 6, 7, and 8 [74]. In mice, CXCR2 binds the CXCL ELR$^+$ chemokines CXCL1, CXCL2, and CXCL6 [74].

Neutrophils are mainly recruited through a gradient of CXC chemokines that are recognized by their surface receptors CXCR1 and CXCR2, which are receptors associated with inflammatory conditions [75]. These receptors bind to chemokines from the CXCL8/IL-8 family whereas in mice they bind to their homologues, the chemokines CXCL1, CXCL2 and CXCL6, which are chemoattractant mainly to neutrophils [76]. Chemokines were only recognized as chemotactic after the characterization of IL-8, which was discovered because of its association to inflammation and because it had an important role in the migration of neutrophils in chemotactic essays [77].

The receptors CXCR1 and CXCR2 are expressed in a variety of cells of the immune system and they are quickly increased during the inflammatory process [75]. In different experimental animal models of arthritis were neutrophils have been depleted or CXCR1/2 has been blocked with an allosteric competitor there is a reduction of the inflammatory parameters evaluated [34, 36]. In an acute model of AIA and a *S. aureus*-induced septic arthritis model in mice, blockage of CXCR1/2 was able to reduce the hypernociceptive response [34].

CXCR1 and CXCR2 bind CXCL6 and CXCL8; however, CXCR2 can also bind CXCL1, CXCL2, CXCL3, CXCL5 and CXCL7 (Table 1) [78]. These chemokines are commonly named the CXCL8 family. CXCL8 can be released by numerous cell types after stimulation including, endothelial cells, T cells, tumor cells, epithelial cells, neutrophils, monocytes, synovial cells, keratinocytes, hepatocytes and fibroblasts (Table 1) [75]. CXCR1/2 are expressed on neutrophils, monocytes, mast cells, basophils, NK cells and CD8 T cells. However other cells besides leukocytes can express these receptors, including fibroblasts, neurons, endothelial cells, hepatocytes, smooth muscle cells and keratinocytes. In non-lymphocyte cells, activation of this receptor may contribute to angiogenesis and tumor growth (Table1) [75]. In these cells, activation of the receptors may contribute to many actions including angiogenesis and consequent tumor growth [79]. Other neutrophil receptors can play important roles in cell migration and subsequent disease severity. CCR2, a receptor for CCL2, was found to be overexpressed on neutrophils from RA patients and was

directly related to the detrimental infiltration of neutrophils into the joints in patients with RA [80].

Since chemokines are necessary to guide neutrophils towards the injured tissue, blocking their receptor, CXCR2, could therefore be an effective strategy to treat AIA mice along side identifying mechanisms of articular injury caused by the infiltration of neutrophils.

In this study, we used the compound Reparixin (DF1681B), an allosteric non-competitive antagonist of CXCR1/2. Reparixin is a derivate of the phenyl propionic acid and a competitive allosteric antagonist of this receptor [30, 34, 81, 82]. In previous studies were mice that were induced with acute models of inflammation were treated with Reparixin, there was a reduction of the articular inflammatory response, mainly a reduction in the number of neutrophils recruited towards the synovial membrane [30, 81, 82]. In a model of brain ischemia and reperfusion the treatment with Reparixin was linked to the decrease in the recruitment of neutrophils, which was directly correlated with a decrease in the injury caused after the reperfusion[30]. This protective result in the case of reperfusion after ischemia was also observed in a model of traumatic spinal injury, where the blockade of the CXCR1 and 2 receptors was successful in preventing further damage [82].

Leukocyte population	Chemokine expression	Receptor expression
Neutrophils	CXCL1/GRO-a, CXCL2/GRO-b	CXCR1/CXCR2
Monocytes	CXCL1/GRO-a, CXCL8	CXCR1
T cells CD8+	CXCL8	CXCR1/CXCR2
Mast cells		CXCR1/CXCR2
Basophils		CXCR1/CXCR2
Natural killer cells		CXCR1/CXCR2
Myeloid-derived suppressor cells		CXCR1/CXCR2
Macrophages	CXCL5/ENA-78, CXCL8	
T cells	CXCL1/GRO-a, CXCL2/GRO-b, CXCL3/GRO-g	CXCR1/CXCR2
Eosinophils	CXCL1/GRO-a, CXCL5/ENA-78	
Non leukocyte population	**CXC chemokine expression**	**Receptor expression**
Keratinocytes	CXCL8	CXCR1/CXCR2
Fibroblasts	CXL5/ENA-78, CXCL8	CXCR1/CXCR2
Epithelial cells	CXCL1/GRO-a, CXCL5/ENA-78, CXCL8	CXCR1/CXCR2
Hepatocytes	CXCL8	CXCR1/CXCR2
Synovial cells	CXCL8	
Endothelial cells	CXCL1/GRO-a, CXCL3/GRO-g, CXCL5/ENA-78, CXCL6/GCP-2, CXCL8	CXCR1/CXCR2
Neurons		CXCR1/CXCR2
Melanocytes		CXCR1/CXCR2
Smooth muscle cells	CXCL1/GRO-a, CXCL2/GRO-b, CXCL3/GRO-g	CXCR1/CXCR2
Tumor cells	CXCL1/GRO-a, CXCL2/GRO-b, CXCL3/GRO-g, CXCL8	CXCR2
Oligodendrocytes	CXCL1/GRO-a	CXCR2
Trophoblasts	CXCL2/GRO-b, CXCL3/GRO-g, CXCL6/GCP-2, CXCL8	
Endometrial cells	CXCL2/GRO-b	

Table 1. Cell expression of agonists and chemokine receptors from IL-8 family. Obtained from Russo et al. 2015 [75].

2. HYPOTHESIS

The main functions of neutrophils are associated with their activity in the innate immune response. However, recent studies have established that they have a role in the setting of the adaptive immune response, mainly considering their potential role in the activation of T lymphocytes, even as antigen presenting cells. Different chemokines and chemokine receptors can activate and promote the recruitment of neutrophils. However, the main receptors that perform this function are CXCR1 and CXCR2, which recognize the ligands of the CXCL8/IL8 family, and their homologues in mice, the chemokines CXCL1, CXCL2 and CXCL6. The hypothesis of this work is that neutrophils develop an important role mounting the immunoarticular response in a known model of prolonged inflammatory antigen-induced arthritis in mice. Therefore, we hypothesize that the activation is associated both with the innate and the adaptive immune response. Even more, we consider that blocking the CXCR2 receptor by treating with a non-competitive allosteric antagonist, reparixin (DF1681B), will modulate the functions of neutrophils in this model, inhibiting the development of arthritis, which would lead to a reduction of the articular pain and the knee tissue damage characteristic of this model of prolonged arthritis.

3. OBJECTIVES

3.1 Main objective

Study the capacity of a CXCR1/2 allosteric inhibitor to block neutrophil influx, neutrophil activation and articular inflammation in a model of prolonged inflammatory antigen-induced arthritis (AIA) in mice.

3.2 Specific objectives

- Evaluate the kinetics of neutrophil migration to the joint in a model of prolonged inflammatory AIA in mice.

- Evaluate the expression of MHCII and CD86 on neutrophils that migrate into the knee cavity, draining lymph node and spleen of mice induced with a model of prolonged inflammatory AIA in mice.

- Evaluate the expression of MHCII and CD86 on neutrophils and their recruitment in to the knee cavity, draining lymph node and spleen of mice induced with a prolonged inflammatory AIA model after blocking the CXCR2 receptor with Reparixin.

- Evaluate the hypernociceptive response and the tissue damage through histology analysis in mice induced with a prolonged inflammatory AIA model after blocking the CXCR2 receptor with Reparixin.

4. METHODS

4.1 Biologicals and reagents

C57BL/6J male mice aged eight-to-ten-weeks were acquired from the Central Bioterium at the Federal University of Minas Gerais (UFMG) and transferred to our own facilities at the Immunopharmacology Laboratory (ICB-UFMG). Animals were fed commercial laboratory chow and allowed to drink filtered water *ad libitum*. Throughout the experimental procedures, mice were kept in a controlled environment at 25 °C and a 12/12 h light–dark cycle. All procedures conducted received prior approval by the animal ethics committee of the UFMG (cetea 86/2014).

The non-competitive allosteric CRCX1/2 inhibitor, Reparixin DF1681B (*R*(–)-2-(4-isobutylphenyl) propionyl methane-sulphonamide) was kindly provided by Dompé Pharma-Italy. Reparixin was dissolved in saline and administered orally in a 15 mg/kg concentration, 100 μl, every 24 hrs.

Methylated bovine serum albumin (mBSA), Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA) and *Bordetella pertussis* toxin were bought from Sigma, St. Louis, MO. Killed *Mycobacterium tuberculosis* desiccated H37Ra was bought from BD Difco Adjuvants.

4.2 Experimental model of Prolonged Inflammatory Antigen Induced Arthritis (AIA)

The prolonged AIA mice model takes 52 days to be elicited, and was induced by administering 2 intradermal immunizations with mBSA, followed by 2 intra-articular challenges with the same antigen [29, 31].

On day –52, mice were subjected to anesthesia (80 mg/Kg of ketamine and 15 mg/Kg of xylazine, intraperitoneally (i.p.) injection) and immunized intra-dermally (i.d.) at the base the tail with 100 μg of mBSA in 100 μl of an emulsion 1:1 of saline and CFA, which was supplemented with 4 mg/mL of killed *Mycobacterium tuberculosis*.

One hour before the first immunization an intra-peritoneal injection of 200ng of *Bordetella pertussis* toxin was administered.

Seven days after the first immunization (day –45), a second immunization was performed, were instead of CFA, IFA is used and it was not supplemented with killed *M. tuberculosis*.

Fourteen days later (day –30) mice placed under anesthesia were challenged with an intra-articular injection (i.a.) of 100µg of mBSA in 10µl of saline, into the tibio-femoral joint.

Thirty days later (day 0), mice placed under anesthesia were given the second i.a. challenge with another injection of 100µg of mBSA in 10µl of saline.

In the 5th, 10th and 15th day after the second challenge the mice were killed for analysis (Figure 2).

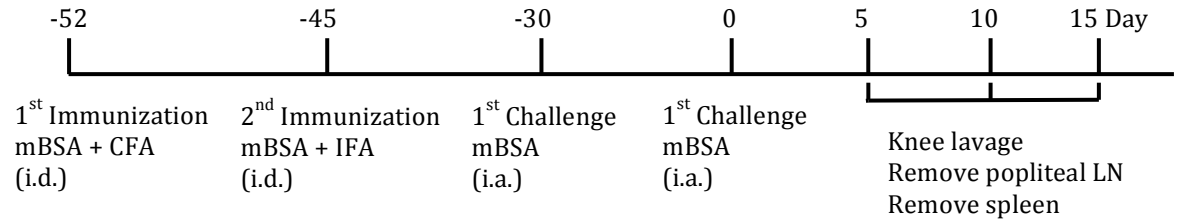

Figure 2. Flowchart of the prolonged inflammatory AIA model.
The prolonged inflammatory AIA model was developed as follows: at day –52: primary i.d. immunization of mice using mBSA emulsified in CFA; at day –45: re-immunization with mBSA emulsified in IFA; at day –30: 1st i.a. challenge with mBSA and then at day 0: 2nd i.a. challenge with mBSA. On the 5th, 10th and 15th days after the 2nd challenge, mice were sacrificed for analysis of different parameters.

4.3 Evaluation of the inflammatory process

On day 5, 10 or 15 after the 2nd challenge, the knee articular cavity was washed with 10 µl of BSA 3% in saline buffer, to collect the cells recruited to the intra-articular space. The number of cells was determined staining with Turks solution and counting in a Neubauer chamber. Then, samples were subjected to analysis by fluorescence-activated cell sorting (FACS). The peri-articular tissue was removed to analyze

cytokine, chemokine and MPO production. The popliteal lymph node and spleen were also removed, processed and analyzed by FACS.

4.4 Evaluation of the participation of CXCR2 in the inflammatory process

To determine the role of CXCR2 in the inflammatory response elicited by the prolonged AIA model, mice were treated orally with Reparixin DF1681B (15 mg/kg diluted in 100µl saline). Treatment started from the 5th (T1) or from the 7th day (T2) and was given every 24 hours until euthanasia was performed. Control mice received 100 µl of saline (Figure 3).

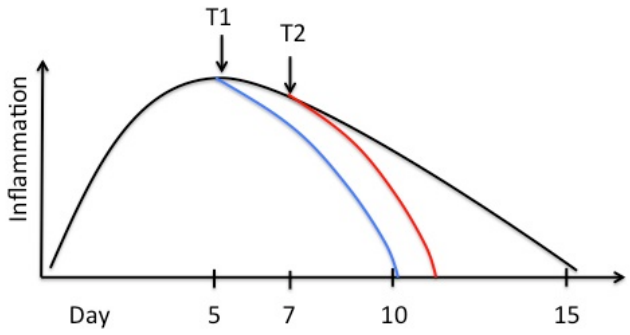

Figure 3. Inflammatory response in the AIA model after treatment with Reparixin.
AIA mice were treated with Reparixin, an allosteric non-competitive antagonist of CXCR1/2 receptors. Reparixin was dissolved in saline and administered orally at 15mg/kg in 100µl every 24hrs. Two therapeutic regimes were followed: Treatment 1 (T1) started on the 5th day Post-challenge (PCh), until euthanasia. Treatment 2 (T2) begun on the 7th day PCh, until euthanasia. On days 5, 10 or 15 PCh, mice were euthanized and tissues evaluated for several inflammatory parameters. The expected inflammation curves after T1 (blue) and after T2 (red) are shown.

4.5 Knee joint evaluation

After induction of prolonged AIA, mice were euthanized administering an overdose of anesthetic (240 mg/Kg of ketamine and 45 mg/Kg of xylazine, intraperitoneally (i.p.) injection) on the 5th, 10th or 15th day PCh, as indicated.

4.5.1 Knee lavage

At the indicated time points, the knee cavity was washed 2 times with 5μL of saline plus 3% BSA and the obtained cells were diluted in 90μL of saline plus BSA 3%. Leukocytes were stained with Turks solution and counted with an optic microscope using a Neubauer chamber to obtain total numbers. Differential counting was obtained from cytospin preparations (Shandon III; ThermoShandon, Frankfurt, Germany) stained with May–Grunwald–Giemsa. Leukocytes were also analyzed via FACS to obtain the total number of cells and to analyze surface protein expression.

4.5.2 Removal of knee tissue and processing

Following lavage, the knee tissue surrounding the cavity was removed surgically and weighted. This was prepared to use in immuno-enzymatic assays as follows: The tissue was homogenized (TissueLyser II – Qiagen) in a solution of cytokine extraction containing protease inhibitors (0.4 M NaCl, 0.1 mM Phenylmethylsulfonyl fluoride, 0.1mM Benzethonium chloride, 10 mM EDTA, 0.05% Tween 20, 0.5% BSA, 20 μl Aprotinin (Sigma)), using 1mL per 100mg tissue. After homogenization, the sample was centrifuged at 12.000 xg for 10 min at 4 °C. The supernatant was collected and stored at –20 °C for cytokine quantification while the pellet was re-suspended in phosphate buffer with 5% hexadecyltrimethyammonium bromide (*HETAB*) and re-homogenized to measure myeloperoxidase activity.

4.6 Measurement of chemokines, cytokines, and myeloperoxidase activity (MPO)

4.6.1 Analysis of chemokines and cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatant obtained from knee samples was processed and used to perform cytokine and chemokine analysis for CXCL1, IFN-γ and IL-10. In all cases the adequate ELISA kit was used in accordance with manufacturer instructions (R&D Systems, Minneapolis, MN, USA).

A 96 well plate (NUNC Thermo Scientific) was coated with capture antibody diluted in PBS, sealed and incubated overnight at 4°C. The plate was washed 4 times with 300 µL of wash buffer (PBS/Tween 0.5 %) using an automatic plate washer. The plate was blocked with 100 µL of blocking buffer (PBS/ 1% BSA), incubated for 2 hours at 37°C and washed. Samples and standards were diluted to the desired concentrations and 50 µL aliquots were added to each well. The plate was sealed, incubated for 2 hours at room temperature (RT) and washed. The detection antibody was added to the plate (100 µL per well). The plate was sealed, incubated for 2 hours at RT and washed. Then 50 µL Streptavidin-Horseradish Peroxidase Conjugate (HPR) 1:200 per well was added. The plate was sealed, incubated for 30 minutes at RT in a dark room and washed. Then 50 µL OPD substrate per well was added. The plate was incubated for 20-30 minutes at RT in a dark room. The reaction was stopped adding 25 µL per well of a solution of H_2SO_4 1M. After stopping the enzymatic reaction, the plate was read at 490 nm, using an ELISA plate reader (SpectraMax Plus microplate reader – Molecular devices).

4.6.2 Myeloperoxidase (MPO) activity analysis

The pellet obtained from the processed knee samples was used to assay for MPO activity as described earlier [83].

Polycarbonate test tubes containing the homogenized knee tissue were subjected to three consecutive cycles of immersion in liquid nitrogen (snap freeze) and immediate removal and thawing at room temperature. Then, samples were centrifuged and the supernatant was collected. MPO activity was measured using 1.6 mM TMB and 0.5 mM H_20_2 in a SpectraMax Plus Microplate Reader (Molecular Devices) measuring O.D. at 450 nm. Results were expressed as relative units.

4.7 Removal and processing of the popliteal lymph node and the spleen

In addition to the removal of the knee tissues, the popliteal lymph node and the spleen were removed surgically and were used to analyze leukocytes by flux cytometry (Figure 4).

4.7.1 Lymph node

The popliteal lymph node (LN) was placed in 1mL of complete RPMI medium (RPMI 1640 + 10 % heat-inactivated bovine fetal serum (BFS), Thermo Fischer, MA, USA). LN was macerated and centrifuged at 1100 rpm at 4°C for 10 minutes. The pellet was re-suspended in complete RPMI medium. Cells were stained with Turks solution and counted under an optic microscope using a Neubauer chamber to determine the total number of leukocytes and verify their viability. Leukocytes were analyzed via FACS to obtain the total number of cells and to analyze surface protein expression.

4.7.2 Spleen

The spleen was collected in a tube containing 10mL RPMI 1640 medium (Thermo Fischer, MA, USA). The spleen was macerated and centrifuged at 1100 rpm, 4°C for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 9 mL of distilled water to lyse the cells, and immediately 1mL PBS 10X was added. The tube was centrifuged at 1100 rpm, 4°C for 10 minutes and re-suspended in 2 mL RPMI 1640 medium. Cells were stained with Turks solution and counted under an optic microscope using a Neubauer chamber to calculate the total number of leukocytes and their viability percentage. Leukocytes were analyzed via FACS to

obtain the total number of cells and to analyze surface protein expression.

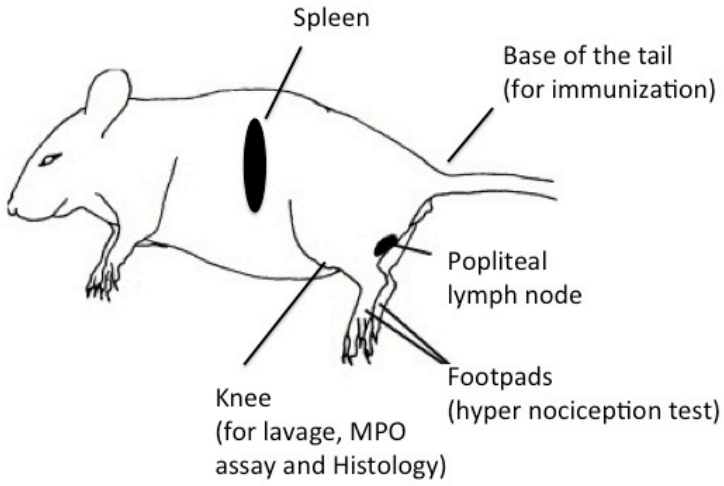

Figure 4. Knee, spleen and popliteal lymph node of AIA mouse.
After induction of prolonged AIA, mice were euthanized by anesthetic overdose on the 5th, 10th and 15th day post-challenge. The knee was used for lavage of the articular cavity, to obtain tissue for ELISA and for MPO activity assays. Otherwise, samples were used for histological analysis. The popliteal lymph node and the spleen were surgically removed and used to analyze leukocyte populations by flux cytometry. In a different experiment, mice were induced with prolonged AIA, and the hyper nociceptive response was evaluated every 2 days by applying pressure to the hind paw.

4.7.3 Splenocyte culture

The spleen was removed, macerated and the splenocytes were obtained as explained before. After the cells were counted in an optic microscope, a 24 well cell culture plate was coated with 10^6 cells in RPMI and incubated at 37ºC with mBSA. After 48 hours of incubation, the content of each well was collected and centrifuged at 1200 g, 10 minutes at 4ºC. The supernatant was collected and used to measure IL6, IL17 and IL10 cytokines through ELISA.

4.8 Fluorescence-activated cell sorting (FACS) analysis

Leukocytes were obtained from the knee articular cavity, the popliteal lymph node or the spleen as described earlier, and were analyzed by FACS. Samples were analyzed using a flux cytometer (FACScan, Becton Dickinson, USA) and data analysis was performed using Flow Jo X10.0.7v software (Tree Star, USA).

Through a forward *versus* side scatter (*FSC vs SSC*) gating analysis, the distribution of cells in the light scatter based on size and intracellular composition, respectively, was characterized. Also, the debris was separated from the lymphocytes. From these lymphocytes, the subset that expressed the marker for neutrophils, Ly6G+, was isolated (Figure 5). This subset was analyzed for the expression of the markers for MHCII+ and CD86+. Markers used to separate TCD4+ lymphocytes were CD3+ CD4+. Then, to determine which were activated CD44+ was used (Figure 6). The antibody combinations for the experiments were: Ly6G/Pacific Blue-A, MHCII PE-Cy5, CD86/APC-A, and CD3/PE-Cy7 CD4/FITC-A CD44.

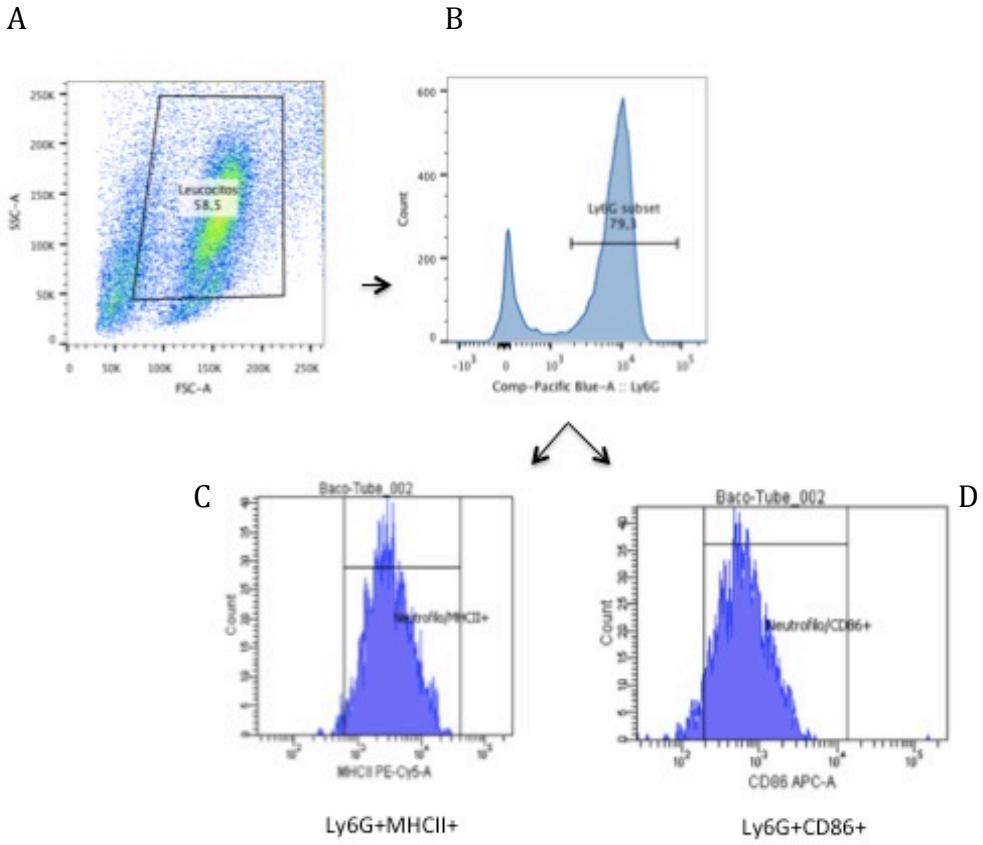

Figure 5. Gating strategy for neutrophils expressing MHCII and CD86.
(A) Through an FSC versus SSC analysis, leukocytes were selected based on size and granularity. (B) Neutrophils were selected based on the expression of Ly6G+. (C) Neutrophils expressing MHCII were considered as Ly6G+ MHCII+, (D) neutrophils expressing CD86 were considered as Ly6G+ CD86+, while neutrophils expressing both MHCII and CD86 were considered as Ly6G+ MHCII+ CD86+ (not shown).

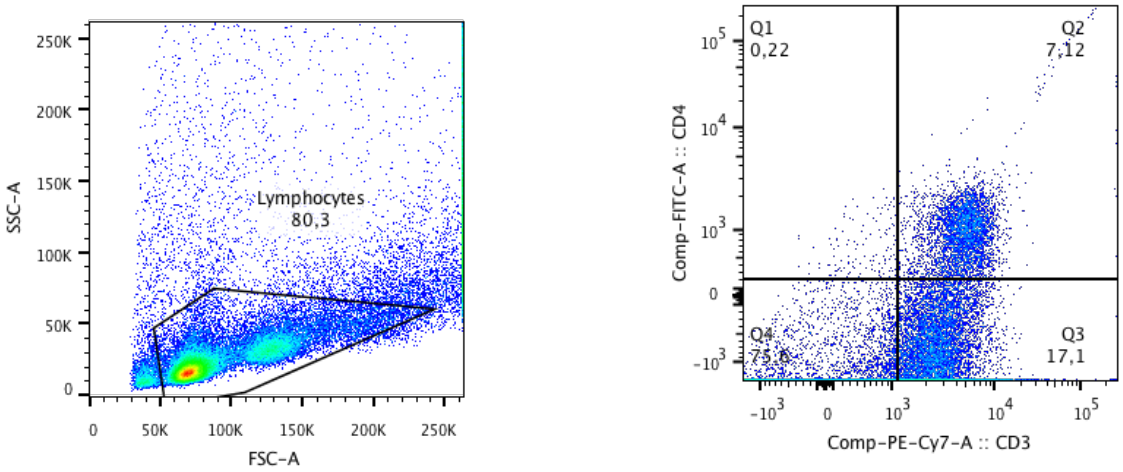

Figure 6. Gating strategy for TCD4⁺ cells.
Through an FSC versus SSC analysis, (A) leukocytes were selected based on size and granularity. (B) TCD4⁺ cells were defined as CD3⁺ CD4⁺. Activated TCD4⁺ cells were defined as CD3⁺ CD4⁺ CD44⁺ (not shown).

4.9 Evaluation of hypernociception

Mechanical hypernociception was measured as previously described [34], using an electronic pressure meter (INSIGHT Instruments, Brazil). After applying perpendicular pressure to the hind paw to induce dorsal flexion of the tibio-femoral joint, the pressure meter automatically records the force applied when the paw is withdrawn. The flexion-elicited withdrawal threshold was used to infer behavioral responses associated with pain. Results express the withdrawal threshold (in grams). Hypernociception was evaluated every 24 hours post-challenge. For adaptation to the surroundings, before the experiment mice were left for 15 minutes inside the acrylic boxes where the experiment was to be performed. Three consecutive stimuli were administered to each mouse and the mean was calculated.

4.10 Histopathologic analysis

After removal, the tibio-femoral joints were fixed in 10% buffered formalin (pH 7.4) for 24hrs, decalcified for 30 days in 14% EDTA, embedded in paraffin, sectioned (thickness 6 μm), and stained with hematoxylin and eosin (H&E).

A single pathologist performed a double blind microscopic examination of two sections per knee joint. The severity of the injury was assessed using histologic parameters previously defined [29], and scored (see below), as follows: a) severity of synovial hyperplasia, b) intensity and extension of inflammatory infiltrate, and c) bone erosion. Scores were:

a) Synovial hyperplasia: (0) without alterations; (1) Focal hyperplasia; (2) Extensive hyperplasia; (3) Generalized hyperplasia / Loss of epithelium.

b) Inflammatory infiltrate: (0) Absent; (1) Low or 1- 20%; (2) Mild or 21-40%; (3) Intense or 41 to 70%; (4) Very intense or >71%.

c) Bone erosion: (0) Without alterations; (1) Sporadic gaps; (2) Significant areas of bone loss.

Scores obtained for each parameter were added to obtain an arthritis index (ranging from 0 to 9). Images of the joint surface from each sample were digitalized and evaluated using Image J software (National Institutes of Health, Bethesda, MD).

4.11 Statistical analyses

Data are presented as mean ± SEM, and statistical analysis was performed with 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 and by unpaired test. Results with $p<0.05$ p were considered statistically significant.

5. RESULTS

5.1 In the prolonged inflammatory AIA murine model, the neutrophilic recruitment is intense at day 5 post challenge, returning to basal level at day 15 post challenge.

Rheumatoid Arthritis is a chronic, autoimmune disease that causes joint pain and damage often leading to severe disability [6]. Current treatments involve treating pain and inflammation to prevent further damage, however efforts to find a cure is an urgent need [84]. Here, we focused on a strategy to inhibit neutrophil migration via CXCR2 blockade in an experimental model of prolonged arthritis in mice. We used the compound Reparixin, a non-allosteric antagonist of CXCR2 which has been reported to block the inflammatory process and tissue damage in different models, including brain ischemia and reperfusion [30], traumatic injury to spinal cords from rats [82] and hepatic diseases [85].

The analysis of cell migration to the joint were performed on the 5th, 10th and 15th day post challenge. In regard to the total number of recruited cells, at day 5, there was an increase in the number of cells recruited to the knee cavity (Figure 7A), where a large proportion was identified as neutrophils (Figure 7B) when compared to control group. Later in the inflammatory process, the number of cells decreased, as we gathered less cells at 10th day, and even less at 15th day (Figure 7A and B). The number of cells collected at 15th day had decreased back to a cell number similar to the control group, suggesting that inflammation had been resolved at this time point.

To further analyze the inflammation process, the tissue surrounding the knee capsule was removed, processed and tested for myeloperoxidase (MPO) activity, which is associated with neutrophil activation [83]. MPO activity increased by the 5th day and decreased at the 10th day, which correlated with the quantity of neutrophils obtained from the knee cavity (Figure 7C).

Based on the kinetic of neutrophil accumulation and its activation in the joint, it may be suggested that our AIA mouse exhibited a prolonged inflammatory progression when compared to acute AIA model [34], since neutrophil is still

detectable up to 10 days after challenge, in contrast to 2-3 days of inflammation in AIA acute models. In this regard, it is suggested that the intense of inflammatory response is higher in this model, which could be associated with greater joint damage and hypernociception. In addition, it permits a good window of therapy to block neutrophil recruitment and activation using Reparixin and the evaluation its effect on crucial immune response, tissue damage and hypernociception.

Once the AIA prolonged model was validated through the evaluation of cell recruitment, it was decided to determine whether mice presented symptoms compatible with inflammation. Thus, a hypernociception test was performed in control and in AIA mice, on the 5^{th}, 10^{th} and 15^{th} day post challenge. Here, the peak of hypernociception was observed at the 5^{th} day, correlating with the higher accumulation of neutrophils in the joint. However, this response reduced up to day 15, although it failed to resolve in the last day analyzed (Figure 7D).

Together, our results suggest that the peak of the inflammatory response occurred on the 5^{th} day PCh and prolonged inflammation was observed on day 10^{th} PCh while the end of inflammation had occurred by the day 15^{th} day post challenge. With these results, we decided to evaluate other inflammatory parameters on day 5 PCh, where acute inflammation may be modeled and at day 10 PCh, where prolonged inflammation was clearly established.

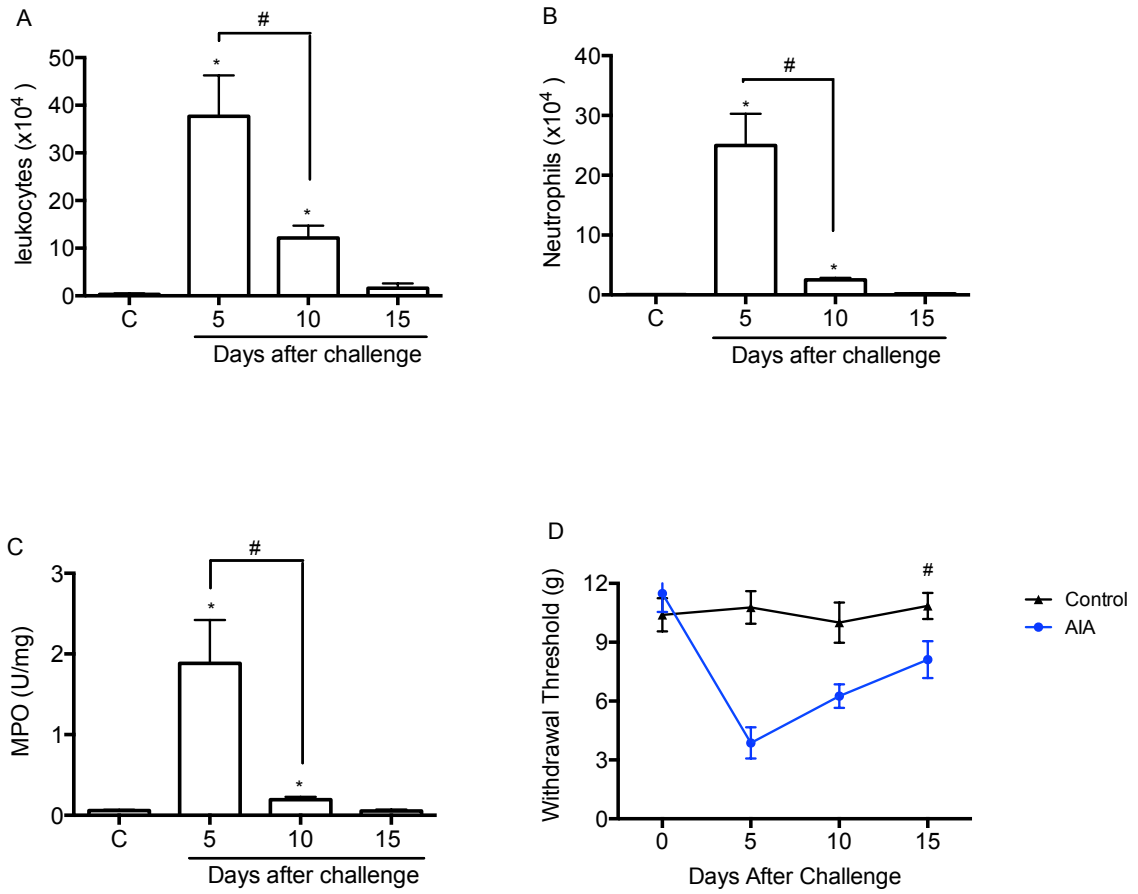

Figure 7. Kinetics of cellular recruitment in the knee articular cavity of AIA mice.
A lavage of the knee articular cavity was made with PBS plus 3% BSA at 5, 10 or 15 days after intra-articular challenge. The lavage fluid was recovered and cells were counted, (A) total cells and (B) proportion of neutrophils. (C) At each timepoint, samples from knee tissue were removed and processed to measure MPO activity. (D) The intensity of hypernociception was evaluated with the paw withdrawal threshold method every 2 days after the challenge, using an electronic analgesimeter. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, #p<0.05 when compared between groups (ANOVA test followed by Newman Keuls test). n = 5–6 mice per group.

5.2 In the prolonged AIA model, MHCII and CD86 expression increases in neutrophils retrieved from the knee articular cavity, the popliteal lymph node and the spleen.

When activated, neutrophils can express antigen presenting cell (APC)-like proteins such as MHCII and CD86 [48], so it was decided to measure their expression as an indicator of neutrophil activation. At day 15 post challenge the amount of cells in the knee joint was too low to further analyze them by FACS. Therefore, all evaluations of neutrophil activation were performed only on the 5th and 10th day post challenge. Neutrophil profile at day 10 was of substantial importance for our analysis as it might be a turning point towards prolonged and ending of inflammation. Both the draining lymph node and the spleen were also removed and used to isolate cells. In these samples, it was determined if during AIA progression, neutrophils were differently recruited to these tissues and whether their activation state was different. Cells from the knee articular cavity, the draining popliteal lymph node and the spleen were collected as described in methods at 5 and 10 days post challenge and analyzed by FACS.

Similar to the results presented in Figure 7, the activation state of neutrophils in the joints also occurred on day 5 post challenge. Neutrophils substantially express MHCII mainly on day 5 (Figure 8A), although the mean fluorescence intensity was similar in day 5 and 10 post challenge (Figure 8B), both higher than control neutrophils. Furthermore, there was also an increase on the number of neutrophils expressing CD86 (Figure 8C), although the intensity of expression of this molecule remained the same (Figure 8D) in all groups. Neutrophils were scattered first by size and granularity and were marked with Ly6G+ as described in methods. Neutrophils expressing MHCII were defined as Ly6G+ MHCII+; neutrophils expressing CD86 were defined as Ly6G+ CD86+ and neutrophils expressing both APC-like proteins were defined as Ly6G+ MHCII+ CD86+. When neutrophils retrieved from the knee cavity expressed CD86, all of them expressed MHCII (Figure 8E). However not all MHCII+ neutrophils expressed CD86+. The expression of both APC-like proteins would allow for neutrophils to have the possibility to present antigen through MHCII. However,

although present in MHCII+ neutrophils, the intensity of the expression of CD86 on neutrophils did not increase during the prolonged AIA model.

As expected, the popliteal lymph node also exhibited an increase in the amount of total cells and neutrophils collected at day 5 post challenge, while a smaller number was still present at day 10 (Figure 9A, B). Consequently the amount of neutrophils expressing MHCII or CD86 increased broadly in day 5 and decreased at day 10 (Figure 9C, E). Moreover, the intensity of MHCII expressed on neutrophils also increased at the peak of inflammation while CD86 remained constant (Figure 9D, F). When analyzing neutrophils obtained from the knee cavity and the lymph node, much more neutrophils were MHCII CD86 double positive at day 5 post challenge when compared to day 10, although both time points were higher than control group (Figure 9G).

In the spleen there was also an increase on the amount of total cells and neutrophils collected at day 5 post challenge (Figure 10A). However, in discrepancy with the data obtained from the knee and the lymph node, the number of MHCII+ (Figure 10A), CD86+ (Figure 10C) and MHCII+ CD86+ (Figure 10E) neutrophils, did not decrease at day 10. Moreover, the intensity of MHCII (Figure 10B) and CD86 (Figure 10D) expression by neutrophils also increased at the peak of inflammation, remaining constant at day 10.

Our results indicated that neutrophil recruitment, as well as the expression of MHCII and CD86 on their surface was similar in the knee joint and in the draining popliteal lymph node. This expression pattern was also present in neutrophils obtained from the spleen, however the variation of MHCII expression was milder from day 5 to day 10 post challenge. In all cases, the intensity of MHCII expression was significantly larger than that of CD86, so it was decided to focus the following experiment on the expression of MHCII.

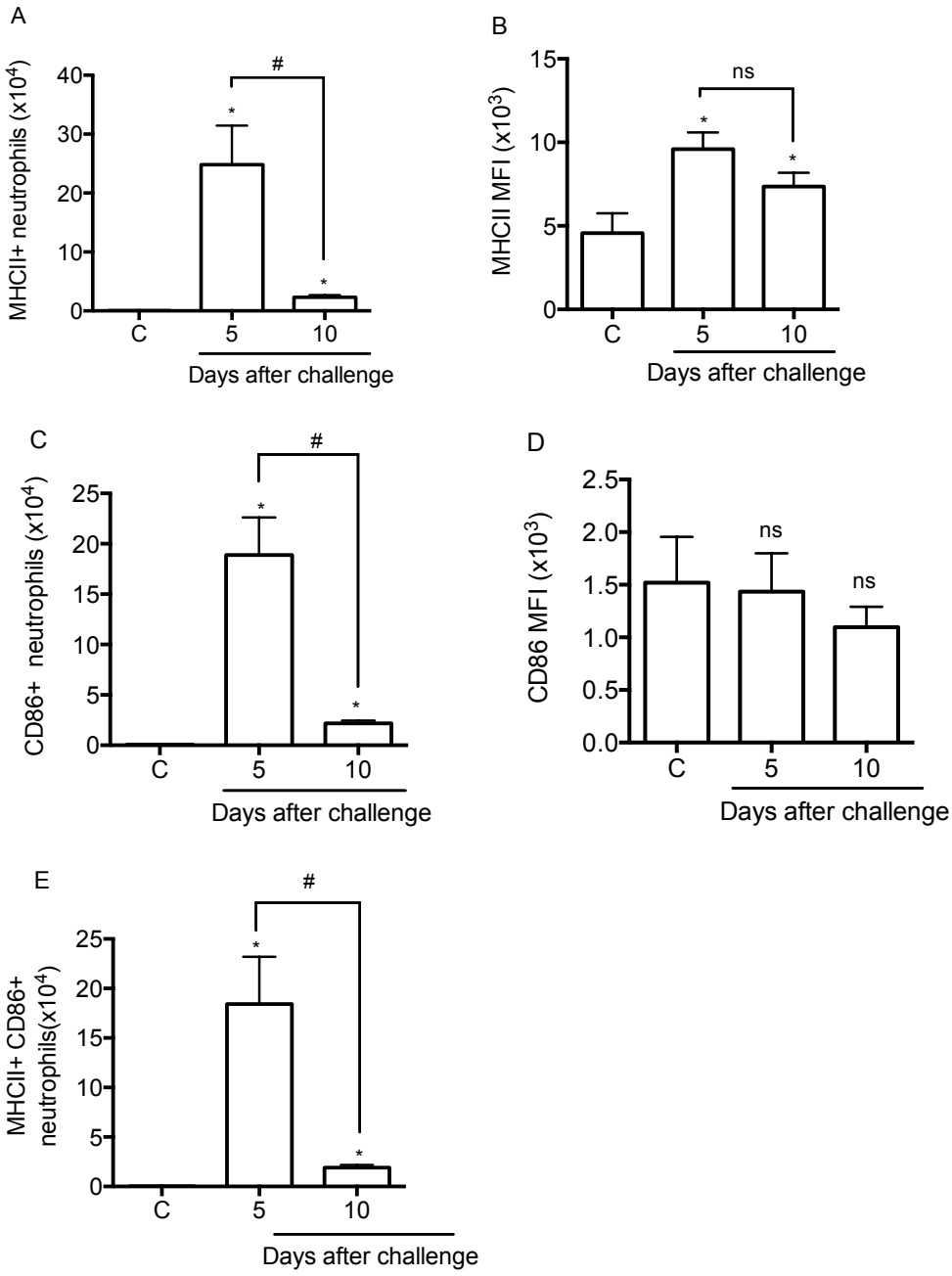

Figure 8. Analysis of CD86 and MHCII expression in neutrophils obtained from the knee articular cavity of AIA mice.

A lavage of the knee articular cavity was made with BSA 3% in PBS at 5, 10 and 15 days PCh. Cells obtained from the lavage were analyzed by FACS to determine (A) the number of neutrophils that expressed MHCII, (B) the mean fluorescence intensity of MHCII in neutrophils, (C) the number of neutrophils expressing CD86 and (D) the mean fluorescence intensity (MFI) of CD86 on neutrophils. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant (ANOVA test followed by Newman Keuls test). n = 5–6 mice per group.

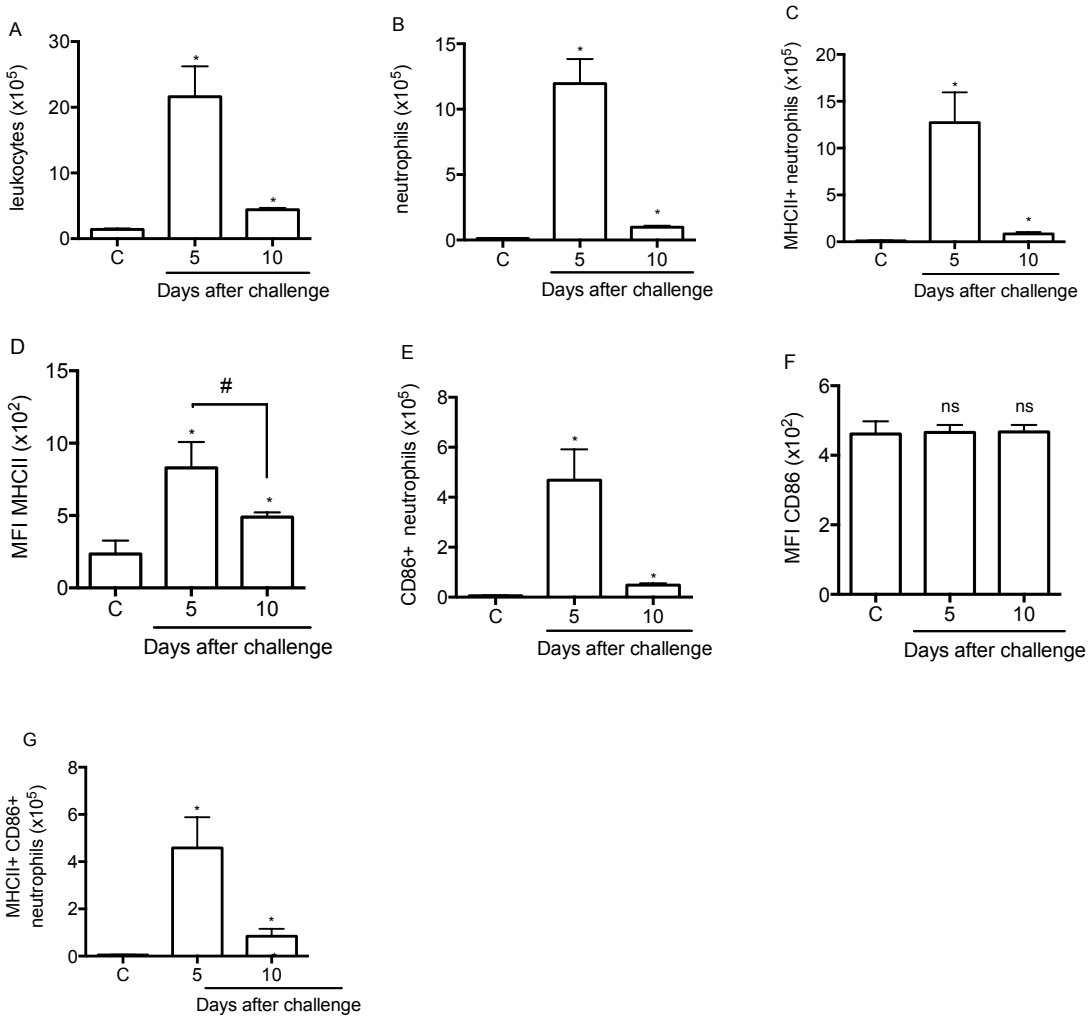

Figure 9. Kinetics of cellular recruitment and analysis of CD86 and MHCII expression in neutrophils obtained from the draining popliteal lymph node of AIA mice.
At 5 and 10 days after intra-articular challenge, the popliteal lymph node of the arthritis-induced leg was extracted and processed to obtain the cells. (A) Total cells and (B) neutrophils were counted. FACS analysis determined the amount of neutrophils that expressed (C) MHCII, (D) MFI-MHCII, (E) CD86, (F) MFI-CD86 and (G) MHCII-CD86. Data are mean ± SEM. *$p<0.05$ when compared with the control group, # $p<0.05$ when compared between groups. ns, non significant. (ANOVA test followed by Newman Keuls test). n = 5–6 mice per group.

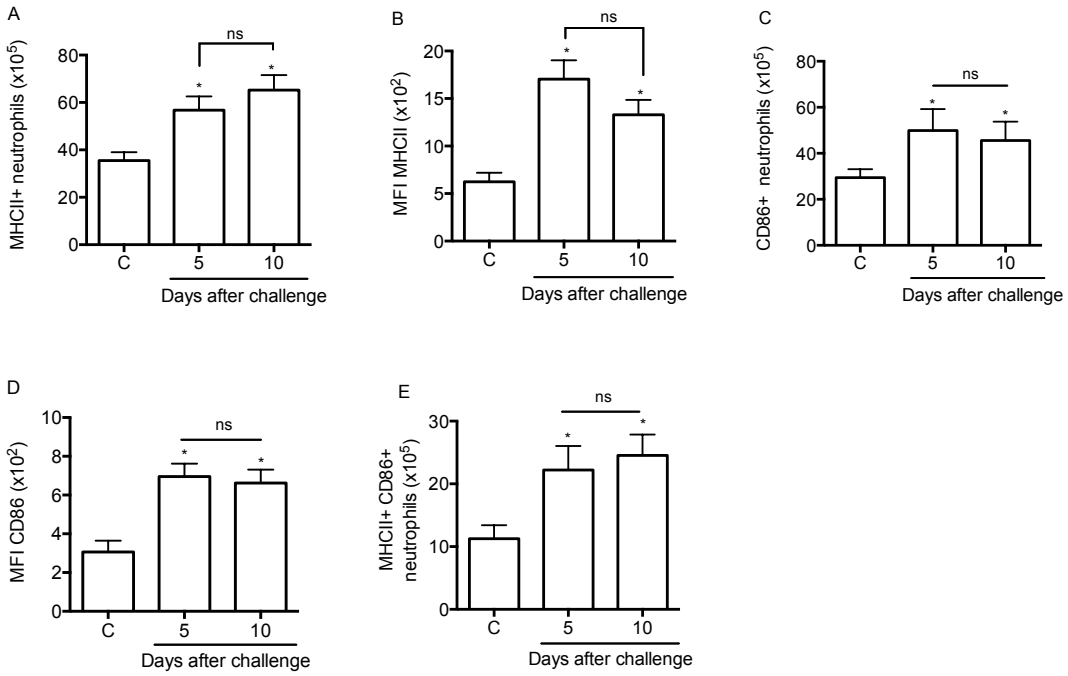

Figure 10. Kinetics of cellular recruitment and analysis of CD86 and MHCII expression in neutrophils obtained from the spleen of AIA mice.

At 5 and 10 days after intra-articular challenge, the spleen was extracted and processed to obtain the leukocytes. FACS analysis was determined the amount of neutrophils that expressed (A) MHCII, (B) MFI of MHCII, (C) CD86 and (D) MFI of CD86 on neutrophils (FALTÓ LA E!!!). Data are mean ± SEM. *$p<0.05$ when compared with the control group, # $p<0.05$ when compared between groups. ns, non significant. (ANOVA test followed by Newman Keuls test). n = 5–6 mice per group.

5.3 Blocking the CXCR2 receptor with Reparixin inhibits cellular recruitment, and decreases the expression of MHCII on neutrophils

The CXCR1/2 receptors are key molecules involved in neutrophil migration during inflammation . As other conventional chemokine receptors, they are G-proteins that may bind different CXC ligands [75]. To evaluate the role of the CXCR2 receptor in the prolonged AIA model, we treated mice with Reparixin, an allosteric non-competitive antagonist of the CXCR1/2 receptors. Reparixin aids in the recovery of inflammatory injuries [82].

The treatment was initiated at day 5 post challenge in an effort to mimic the treatment patterns usually followed in arthritis patients. Two therapeutic regimes were followed: Treatment 1 (T1) was started on day 5 and ended on day 10 post challenge. Treatment 2 (T2) begun on the 7th day and ended on the 10th day post challenge. On 10th day post challenge mice were killed and tissues were evaluated for inflammatory parameters.

Both treatments (T1 and T2) reduced total cell recruitment towards the articular cavity in AIA-induced mice (Figure 11A). T1 was better at reducing neutrophil influx, reaching levels almost as low as the non-treated group (Figure 10B). This suggests that the time point when Reparixin treatment begins was crucial to achieve interference with recruitment of neutrophils to the articular cavity after induction of chronic AIA. T1 was also slightly better than T2 at reducing the increase in MHCII expression (Figure 11C and D).

Both treatments inhibited the recruitment of total cells and neutrophils at the lymph node from the AIA induced mice (Figure 12A, B). Again, T1 proved to be slightly more effective than T2 to decrease neutrophil recruitment and induction of MHCII expression in neutrophils from AIA mice (Figure 12C and D).

Neutrophils are important producers of cytokines, which allows them to modulate the inflammatory response [56, 86]. Both CXCL1 (Figure 13A) and IFNγ (Figure 13B) cytokine production in the periarticular tissue was decreased by both Reparixin treatment regimes. However, blocking CXCR2 did not alter the production of IL-10 (Figure 13C).

From our results above, suggesting that T1 was more effective than T2 at reducing cell recruitment and MHCII expression on neutrophils, it was decided to further evaluate the effects of the T1 regime on the ending of the inflammatory response by conducting histology and hypernociception experiments.

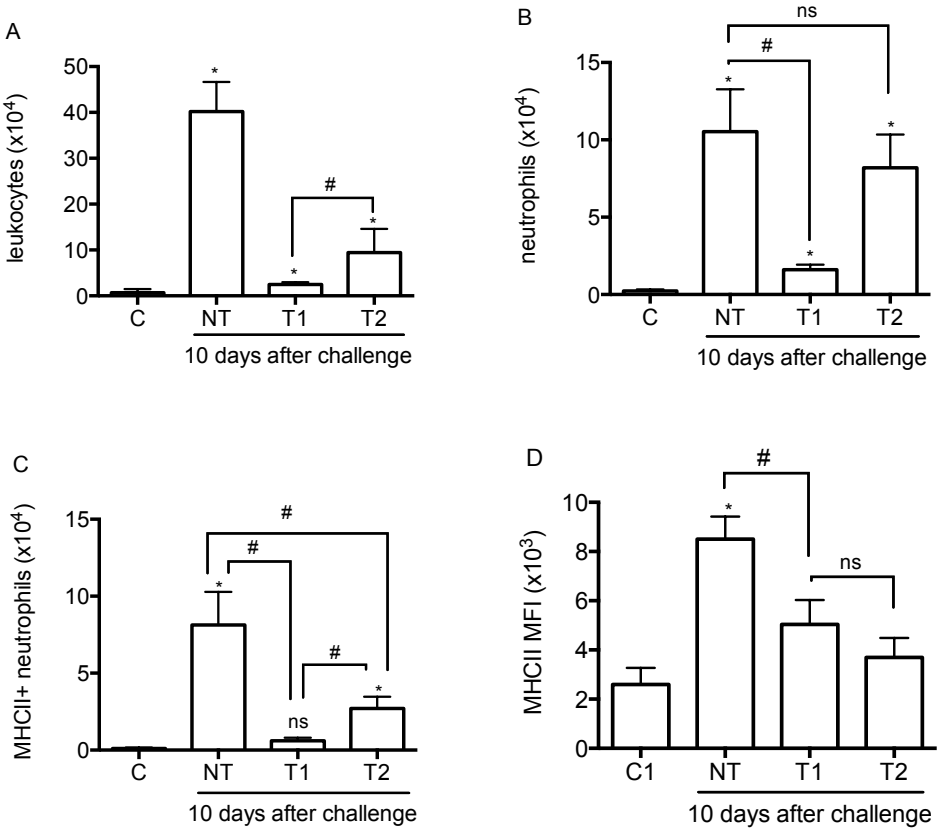

Figure 11. Reparixin blocks neutrophil recruitment into the knee joint and decreases de expression of MHCII on the surface of neutrophils.
Five days after intra-articular challenge, mice were treated orally with Reparixin (T1), every 24hrs up to 10 days after intra-articular challenge. Then, lavage of the knee articular cavity was performed with PBS/ 3% BSA, which was recovered for analysis. (A) Total cell number, (B) neutrophils, (C) total number of neutrophils that expressed MHCII and (D) MFI of MHCII in neutrophils. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant. n = 5–6 mice per group.

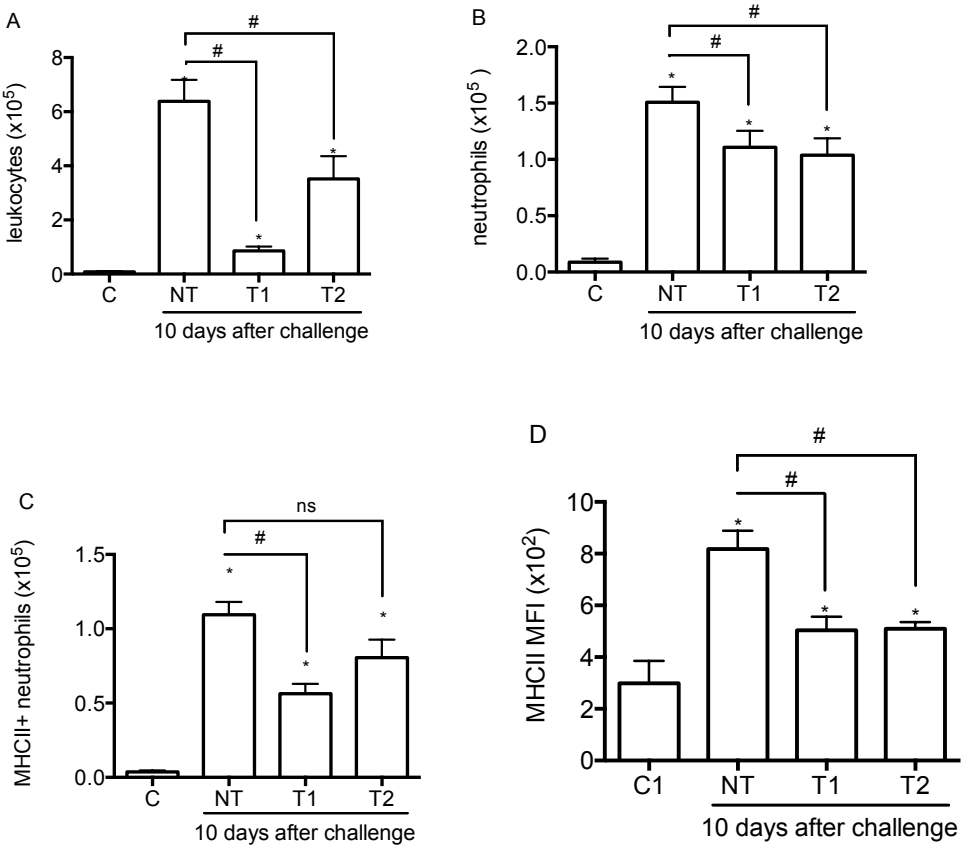

Figure 12. Reparixin blocks neutrophil accumulation in the draining lymph node and decreases the expression of MHCII on the surface of neutrophils.

Five days after intra-articular challenge, mice were treated orally with Reparixin (T1), every 24 hrs. At 10 days after challenge, the popliteal lymph node of the arthritis-induced leg was extracted and processed to obtain the cells, which were analyzed through FACS. (A) total cells (B) total neutrophils (C) number of neutrophils that expressed MHCII and (D) MIF of MHCII in neutrophils. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant. N = 5–6 mice per group.

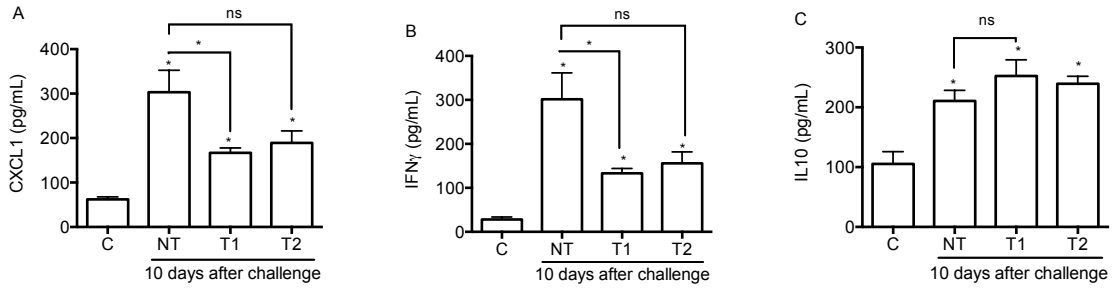

Figure 13. Reparixin decreases the production of pro-inflammatory mediators in mBSA challenged joints.

Five days after intra-articular challenge, mice were treated orally with Reparixin (T1), every 24 hrs. At 10 days after challenge, the knee periarticular tissue of the arthritis-induced leg was removed and processed to test for cytokine accumulation as described in methods. (A) CXCL1, (B) IFNγ and (C) IL10 were quantified by ELISA as described in methods. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant. N = 5–6 mice per group.

5.4 Reparixin ameliorates the mechanic hypernociceptive response and the histopathologic score of prolonged AIA mice

Inflammation mediated damage can be evaluated by observing the histopathological characteristics of the affected tissues and by conducting hypernociception experiments. With this in mind it was decided to analyze the knee joint area from healthy (control mice), AIA mice and AIA mice subjected to treatment protocol 1 (T1).

Histological analysis shows a large cellular infiltrate in the synovial tissue in days 5 and 10 of non-treated AIA groups, with prevalence of polymorphonuclear leukocytes. Other findings included bone erosion, loss of adipocytes, and synovial membrane hyperplasia. None of these was present in the control group (Figure 14A-C and E).

In tissues from AIA mice subjected to protocol treatment 1, (Figure 14C), Reparixin administration led to decreased damage, as decreased cellular infiltration and bone erosion were observed compared to days 5 and 10 of non-treated AIA groups (Figure 14C). Reparixin treatment led to a decrease in the arthritic index as compared to the AIA-non treated mice (Figure 14E). However, the score of the AIA-Reparixin mice still remained elevated in comparison to the control group (Figure 14E). Therefore, it was concluded that Reparixin did diminish articular damage and therefore the arthritic index. However, treatment at the peak of inflammation was not sufficient to prevent damage to the joint, suggesting that most of the damage took place before the peak of the inflammatory response.

To evaluate the recovery in the symptomatology in the AIA mouse subjected or not to Reparixin, the hypernociceptive response was measured every two days up to day 16 post challenge (Figure 15). The hypernociceptive response in AIA mice only started to decrease after the 10^{th} day and failed to total resolve by day 16. Thus, in the prolonged AIA model, although cell recruitment parameters were resolved by day 10, hypernociception did not.

In Reparixin, protocol 1-treated AIA mice, where treatment was started on the 5th day PCh, a decrease in hypernociception was detected as soon as 24 hours later, at the 6th day PCh. Thus, treatment with Reparixin resulted in an immediate effect on the hypernociceptive response (Figure 15). Furthermore, treatment with Reparixin resolved hypernociception in AIA mice by the 12th day PCh (Figure 15).

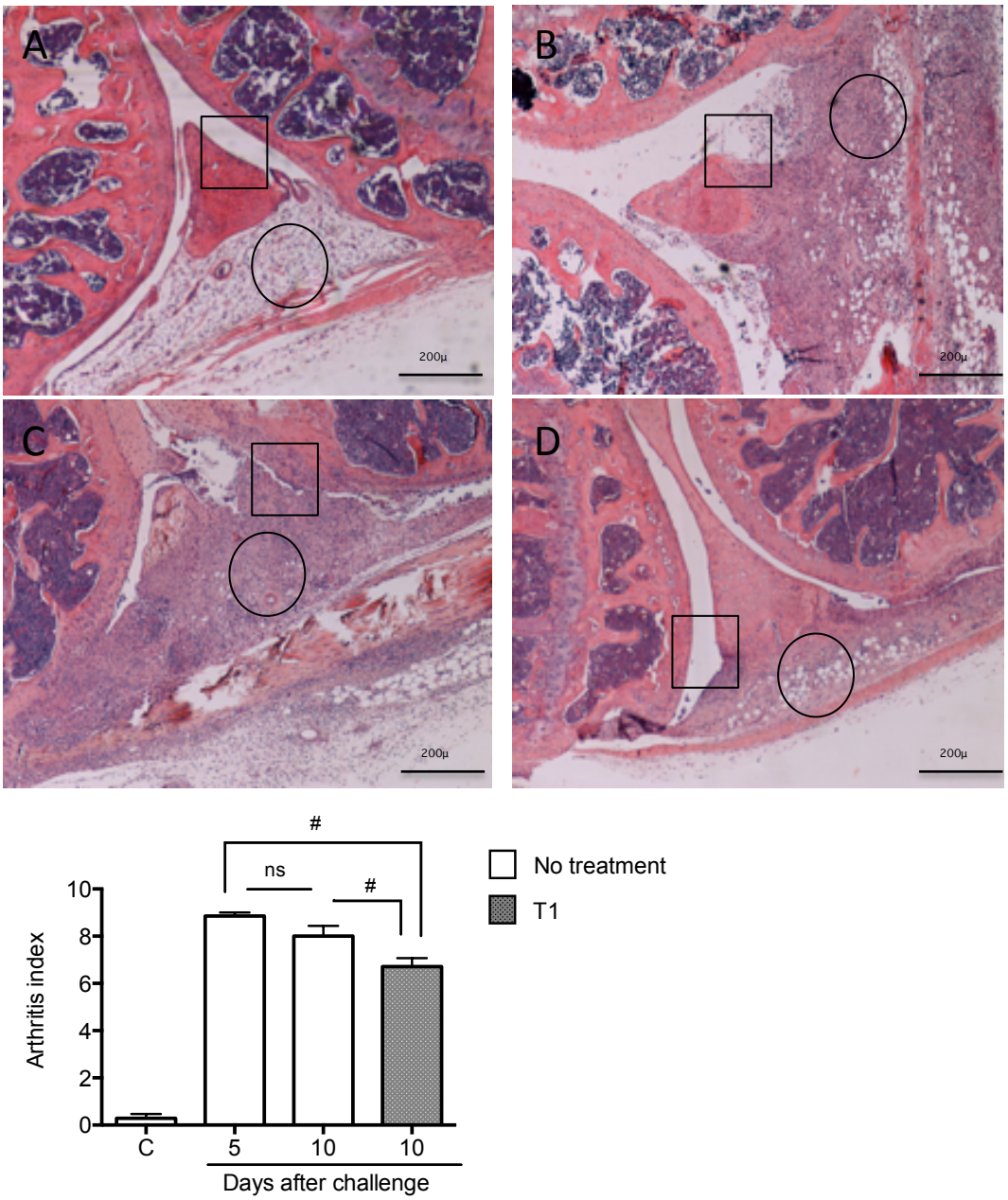

Figure 14. Evolution of articular damage in prolonged AIA. Immunized mice were challenged with mBSA, treated with vehicle or Reparixin, and the knee joints were removed at two different time points (5 or 10 days).

Samples were processed for histopathological analyses. Representative H&E images of (A) control (B) AIA (5 d) (C), AIA (10 d) (D), and AIA (10 d)+Reparixin from day 5 (T1) mice, (circle – cellular infiltrate; square - synovial hyperplasia). Scale bar: 200 µm, as indicated in each image. (E) Histopathological score (arthritis index described in Materials and Methods). Data are shown as mean ± SEM from one representative out of two independent experiments with 24 mice per experiment. # $p<0.05$ when compared to the vehicle-treated group. *N* = 5–6 mice per group.

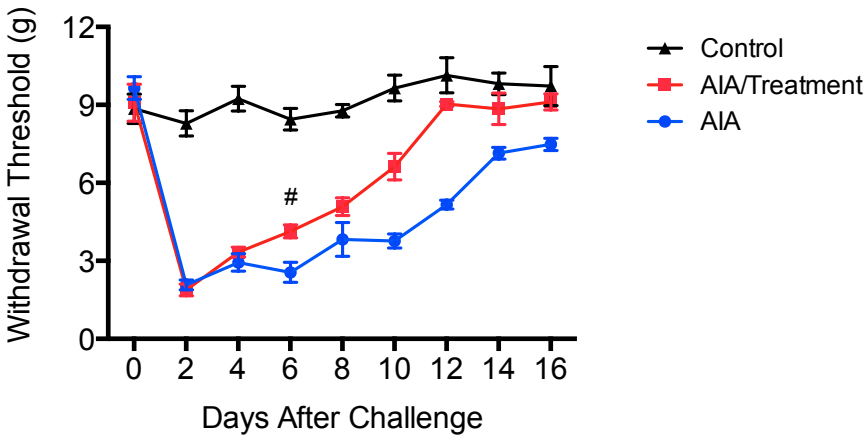

Figure 15. The blockage of CXCR2 decreases hypernociception in the late stages following challenge.
Mice were challenged with mBSA into the knee joint and treatment started 5 days afterwards. The experimental groups were Control: non-treated mice, AIA mice: Challenged mice and AIA/treatment mice: treated daily with Reparixin. The intensity of hypernociception was evaluated determining the paw withdrawal threshold every 2 days after the challenge, using an electronic analgesimeter. Data are shown as mean ± SEM. #$p<0.05$ when compared the Reparixin-Treated group to the non-treated group (t-test followed by unpaired test). N = 5–6 mice per group.

5.5 T cell activation increases during the prolonged inflammatory AIA model

T cell activation located in the inflamed synovial tissue is of great importance in the injury that occurs in this type of arthritis and depends of MHC class II [87]. Therefore we analyzed TCD4 cells obtained from the draining popliteal lymph node. It was found that the amount of TCD4 cells was increased in the lymph node from the AIA mice when compared to control mice (Figure 16A). When the cells were analyzed for the marker of activation CD44, it was shown that control TCD4 cells were not activated while the ones obtained from the AIA mice were (Figure 16B). Also the intensity (MFI) of the expression of CD44 was increased when compared with TCD4 cells obtained from the control (Figure 16C). However treatment from the peak of the inflammatory response did not change the number of activated TCD4 cells (Figure 16B) or the intensity (Figure 16C) of expression of CD44.

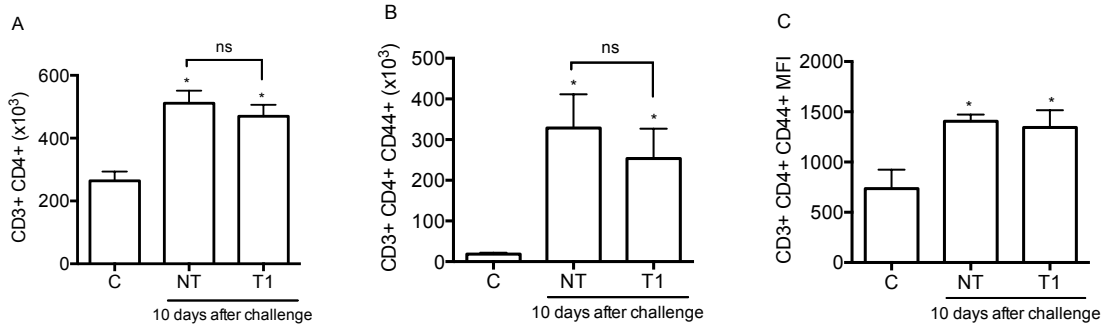

Figure 16. Reparixin block of CXCR2 does not affect T cell accumulation or activation in the draining lymph node.
Five days after intra-articular challenge, mice were treated orally with Reparixin (T1), every 24 hrs. At 10 days after challenge, the popliteal lymph node of the arthritis-induced leg was extracted and processed to obtain the cells, which were analyzed through FACS. (A) Total TCD4+ cells (B) total TCD4+CD44+ lymphocytes, (C) MIF of CD44+ in lymphocytes. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant. N = 5–6 mice per group.

5.6 Splenocytes obtained from AIA mice express proinflammatory cytokines when cultured with mBSA

Splenocytes obtained from the spleen of AIA mice were processed and cultured with mBSA to quantify the cytokine production of these cells after stimulus. Splenocytes from day 5 and day 10 after challenge increased production of IL6, however, it was back at basal levels on the 15th day after challenge (Figure 17A). T1 had no effect on the production of IL6 (Figure 17A). Splenocytes obtained on the 15th day after challenge increased the amount of IL10 they produced after co-culture with mBSA, and T1 had a positive effect on the levels of this cytokine (Figure 17B). Splenocytes from the 5th day after challenge produced more IL17 and slowly decreased throughout the kinetic. T1 did decrease the level of IL17 produced by splenocytes in the 10th and 15th days post challenge (Figure 17C).

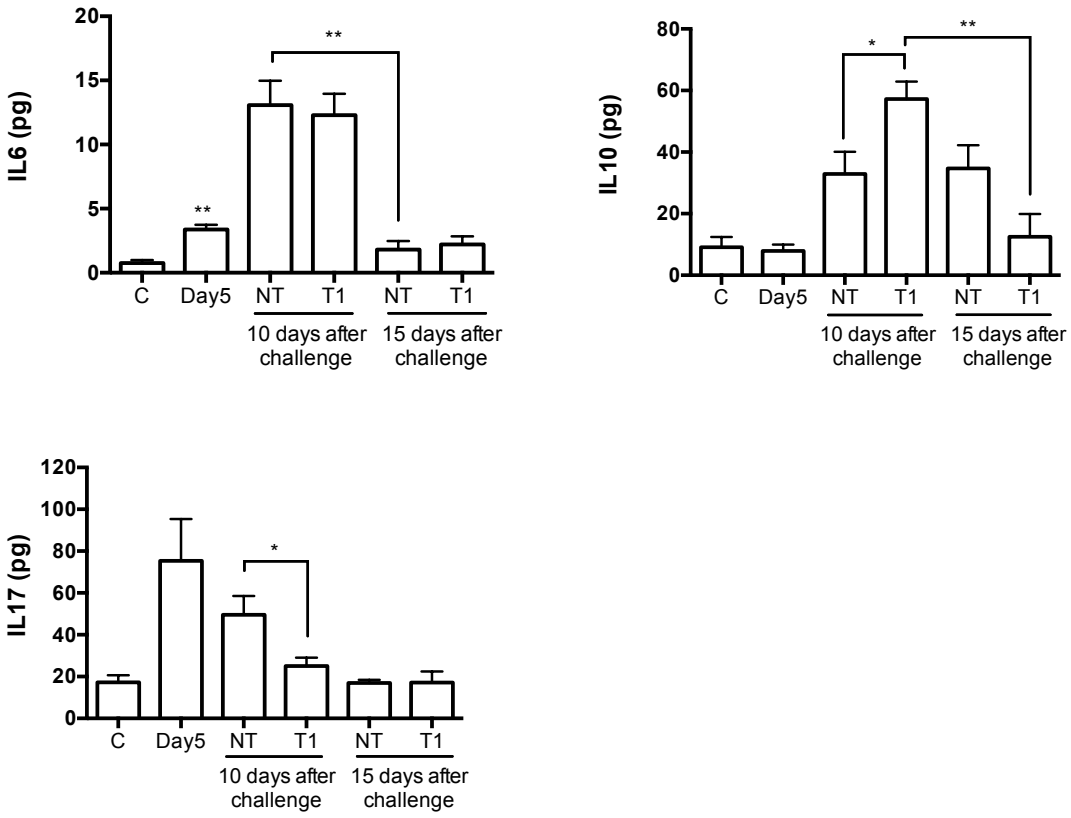

Figure 17. In vivo treatment with Reparixin alters the production of IL-6, IL-17 and IL-10 by splenocytes after stimulation with mBSA.

Five days after intra-articular challenge, mice were treated orally with Reparixin (T1), every 24 hrs. At 5, 10 and 15 days after challenge, the spleen of the arthritis-induced mice was removed, processed and cultured with mBSA to test for cytokine production of splenocytes as described in methods. (A) IL6, (B) IL10 and (C) IL17 were quantified by ELISA as described in methods. Data are shown as mean ± SEM. *p<0.05 when compared with the control group, # p<0.05 when compared between groups. ns, non significant. N = 5–6 mice per group.

6. DISCUSSION

RA is an autoimmune, inflammatory and chronic disease characterized by a large cellular infiltrate, among which neutrophils are crucial for the pathogenesis of the disease [6]. Neutrophils are effector cells that release inflammatory mediators that contribute with chronic inflammation, however neutrophils are also cells with numerous potential roles in the adaptive immune response [6]. Because the main receptors of neutrophils are CXCR1 and CXCR2 [28], the purpose of our work was to study the effect of blocking these receptors in the recruitment and activation of neutrophils in a prolonged inflammatory AIA model, and how this would affect the pain and tissue damage characteristic of the model. Because there is no cure for RA, and current treatments involve treating pain and inflammation to prevent further damage, finding new therapeutic targets is an urgent need [84].

The main findings of our work were:

1. The prolonged inflammatory AIA model caused prolonged inflammation in the joint, tissue damage and hypernociception that where associated with an excessive accumulation of neutrophils in the joint.
2. In the prolonged inflammatory AIA model, the neutrophils obtained from the synovial lavage and the lymph node presented an increase in the expression of MHCII in the peak of inflammation, with a subsequent decrease when the process of resolution started. The expression of CD86 on neutrophils was reduced in the model of chronic AIA.
3. The systemic blocking of CXCR1/2 with reparixin from the peak of inflammation in the AIA model decreases the quantity of neutrophils recruited to the articular cavity and to the lymph node.
4. In the neutrophils collected from mice treated with reparixin, the expression of MHCII was smaller tan when compared with the non-treated mice.
5. Through histology was observed that blocking CXCR1/2 with Reparixin diminishes tissue damage in the knee of mice with AIA compared with non-treated mice.

6. In the mice with AIA, the blocking of CXCR1/2 with Reparixin diminishes the hypernociception.

7. Activation of CXCR1/2 is important for the control of inflammation in the model of prolonged inflammatory AIA.

8. Blockade of CXCR1/2 with Reparixin decreased lymphocyte activation, as seen by decreased production of IFN-g in vivo and IL-17 production by splenocytes.

The mouse model of AIA used in this study caused prolonged inflammation in the joint, associated with a big neutrophil accumulation for prolonged time as compared to other models. Thus, it is a valuable model to study neutrophil phenotype and function in joint inflammation. Neutrophils are important infiltrating cells in RA, and they seem to play a detrimental role in the disease [6, 88]. Although a disease like RA cannot be exactly reproduced in animal models, we could at least mimic punctual characteristics of chronic inflammatory diseases.

The peak of the inflammatory response in our model occurs at day 5 after challenge while by the 15th day it has already finished, at least in the point of view of neutrophil accumulation. This led to us defining the 10th day after challenge as the time point when we could read out inflammatory parameters since we still have joint inflammation, damage and dysfunction. The neutrophils retrieved in this time point were different than control non- activated neutrophils and neutrophils retrieved at the peak of the inflammatory response.

Some studies had already investigated the role of neutrophils and the CXCR1/2 receptors in joint inflammation in a similar, more acute, antigen-induced model of arthritis. The blockade of CXCR1/2 diminishes neutrophil recruitment, which resulted in decreased cytokine production, hypernociception and tissue damage [34]. Furthermore, the depletion of neutrophils with monoclonal antibodies has resulted in decreased joint inflammation and hypernociception [89]. The blockade of chemoattractant receptors or adhesion molecules, which causes a decrease of

neutrophil migration, is also an effective control of inflammation in experimental arthritis [80].

However, we aimed to deepen the comprehension on the role of neutrophils in joint immunopathology, mainly to try to understand how neutrophils could relate to adaptive immunity tissue and cells in this system. To achieve our objectives we used an antagonist of CXCR1/2 receptors called Reparixin [34, 82]. Neutrophils express the CXCR1/2 receptors on the cell membrane, which bind to ELR+ CXC ligands [74]. It is not well established if mice express CXCR1. However, Reparixin is functional in murine models of neutrophilic inflammation acting as a CXCR2 blocker, capable to reduce inflammation in different experimental models [30, 34, 81, 82].

We started the treatment with Reparixin from day 5 or 7 following arthritis induction. These time points have substantial joint inflammation and the idea was to mimic clinical situation by starting a treatment after the manifestation of the disease. At these time points, there is massive accumulation of neutrophils in the joint, which can release granules, enzimes and ROS, important factors that cause tissue damage [88]. In this scenario, there is high production of proinflammatory cytokines, which can lead to an exacerbated immune response that needs to be controlled, making it necessary to find equilibrium between the proinflammatory cytokines and the anti-inflammatory cytokines to avoid irreversible tissue damage to the host [84, 90].

After the articular challenge with mBSA, the chemokines produced recruited neutrophils and mononuclear cells towards the knee joint. These inflammatory mediators have an important role in the pathogenesis of AIA. Among these mediators, the chemokines TNF-α, IL-1β, and IL-17 are related to bone erosion in the joints of patients with RA [91]. The increase on the presence of neutrophils after the AIA onset might be the result of a bigger production of CXCL1 [34, 88]. We observed increased levels of CXCL1 and IFNγ in the periarticular tissue after induction of AIA. These levels were reduced after treatment with Reparixin.

Furthermore, in the model of prolonged AIA, there has been observed an increase in the production of these cytokines in the inflammatory peak of the model

and an increase in the production of IL-10 at a later phase of the model [29]. This is consistent with our findings, were proinflammatory cytokines were produced after challenge and IL-10 was produced in the declining inflammatory phase of the model.

An increase in IL-10 production after induction of chronic AIA was observed. This is in concordance with the increase of IL-10 production in the joints of RA patients and with other studies developed using the chronic AIA model [29]. However treatment with Reparixin had no effect on this cytokine level. IL-10 is an anti-inflammatory cytokine that regulates inflammation and the production of proinflammatory cytokines like TNF-α, IL-1β and IL-6 [92]. IL-10 is augmented in the synovial liquid of patients with RA and in the prolonged AIA model we found an increase in its production. IL-10 is an important cytokine in our model as it was also able to decrease cartilage destruction and bone erosion in a collagen induced arthritis model [93].

Splenocytes obtained from AIA mice that were cultured along with mBSA, presented high levels of IL-6 and IL-17 on their supernatant in the end of the inflammatory response and high levels of IL-10 in the 10th day and 15th day post challenge. In the case of splenocytes from treated mice, the production of IL-6 and IL-17 decreased while the production of IL-10 increases. The cytokine IL-6 posses great relevance in the pathogenesis of RA and in the AIA model, where animals IL-$6^{-/-}$ appeared to be resistant to disease progression [94]. IL-6 is a proinflammatory cytokine, however, studies show also regenerative and antiinflammatory activities [95].

IL-17 is other proinflammatory chemokine produced in the serum and in the synovial fluid of patients with RA. IL-17 has an important role in the pathogenesis of RA as it induces the increase of production of other proinflammatory cytokines like TNF- TNF-α, IL-1β and IL-6, and in a model of AIA the inflammation, pain and bone destruction were reduced after inhibition of IL-17 [96]. We found a reduction of IFNγ production in the lymph node and an increase in the production of IL-17 in the splenocytes. These experiments should be repeated to verify if there is different cytokines expressed in different tissues.

Besides their role in the pathogenesis of inflammatory diseases, neutrophils are also considered plastic cells whose capacity exceeds that of an effector cell. They are viewed as important cells capable of modulating the adaptive immune response and are known to be able to express the APC-like proteins such as MHCII and CD86 in RA, or even act as APCs in vitro [48, 50, 53]. Here, CD86 was constantly expressed on neutrophils and did not suffer major intensity of expression changes. Nonetheless, it was observed that the intensity of the expression of MHCII on neutrophils was vastly increased in neutrophils retrieved from the articular cavity and the lymph node of AIA mice. This intensity is diminished in the 10th day after challenge, but it doesn't reach the same level as the control group. However, the intensity of the expression of MHCII was diminished when this AIA induced mice were treated with Reparixin from the peak of the inflammatory response on. This allows us to propose that CXCR1/2 receptors are associated with the plasticity of neutrophils in the AIA chronic model, specially regarding MHCII expression. This APC like molecule expression on neutrophils could render them the capacity of modulating the immune response through a direct interaction with T cells [97, 98].

The concept that neutrophils could express MHCII and coestimulatory molecules is not new, however it had not yet been tested in the model of prolonged antigen induced arthritis. Based on the kinetic used in this study, we show that the levels of this APC-like molecules changes throughout the progression of the experimental arthritis, and that this correlates with T cell activation and the severity of the symptoms. Neutrophils were proven to be able to consistently present antigen in a MHCII dependent manner to CD4 T cells in vitro [52]. Also, in an ex vivo experiment, neutrophils retrieved from vaccine-draining lymph nodes from rhesus macaques have been able to present vaccine antigen to antigen-specific memory CD4^{+} T cells [52]. Furthermore, neutrophils retrieved from the affected area of a colitis model were found to overexpress MHCII, CD86 and to have obtained the capability to present antigen in a MHCII dependent manner to OVA-specific CD4 T cells [51]. In this same work, it was proposed that neutrophils could be the causing agent behind the activation and proliferation of T cells and the increase in the production of

proinflammatory cytokines leading to gut inflammation to persist and become chronically inflamed [51].

Besides presenting antigen, they have been linked to aiding in the differentiation of T cells towards TH17 and Th1 T cells [53]. Moreover, they have been linked to also being able to cross-prime $CD8^+$ T cells in vivo and to transport antigen from the dermis to the bone marrow to do it [99, 100]. Our results show that neutrophils can acquire the capability of presenting antigen in a MHCII dependent manner after induction of the prolonged AIA model, however the reason why they would do it is not clear. As a result of many autoimmune diseases being T cell dependent, we could suggest that the interaction between neutrophils and T cell somehow activates and promotes T cells into becoming proinflammatory, while at the same time neutrophils can be enhancing the production of proinflammatory cytokines while in the affected tissue or even in the draining lymph node. In other models it has been proven that neutrophils can act as APCs but they don't surpass the ability of antigen presentation of macrophages or dendritic cells. This could mean that the ability of presenting antigen might be for another unknown purpose.

The blocking of CXCR2 in mice, that is highly related to neutrophil recruitment, was also capable of affecting T cells and T cell activation in our experiments. Lymphocytes also express the CXCR2 receptor and the blockade with Reparixin could also affect directly their recruitment. The obvious next experiments for our project would be to test if neutrophils themselves can activate T cells in a major histocompatibility complex class II (MHC-II; HLA-DR)-dependent manner. This could be through ex vivo experiments were neutrophils retrieved from the lymph node could be tested for expression of MHCII, coestimulatory molecules, and their ability to present antigen via MHCII to antigen specific (mBSA) $CD4^+$ T cells ex vivo.

Synovial membrane in RA patients is characterized by an increase in vascularity, cellular hyperplasia and cellular infiltrate, which causes cartilage and bone damage [101]. Sinovial hyperplasia thickens and damages the synovial membrane through the proliferation of the inflammatory cells and the production of inflammatory mediators [101]. Mice with prolonged AIA presented histologic alterations on the tibio-femoral joint. There was an increase in the inflammatory

infiltrate, loss of the integrity of the synovial membrane, reduction of the amount of adipose tissue cells and bone resorption. Meanwhile, the treated mice had less tissue damage, and therefore a smaller arthritic index. This points out a protective effect of Reparixin in prolonged AIA in mice.

Angiogenesis, the formation of new capillaries from existing vasculature is also a recurrent factor that plays a role in the pathogenesis of several autoimmune inflammatory diseases like RA [102]. CXC ELR+ chemokines play an important role in neutrophil migration and angiogenesis, and are more abundant in the synovia of patients with RA [103]. A next step to take would be to study CXCR2-related angiogenesis in our AIA model.

Our results in the protection of tissue can be compared to other models were they have strategized to block CXCR1/2 as well. Blocking CXCR1/2 has been successful to reduce tissue injury in other neutrophil-dependent inflammatory model of brain injury were pretreatment with Reparixin was able to promote neuroprotective effects by reducing PMN infiltration, and tissue damage that is caused after reperfusion [30]. Also in an acute chronic liver failure model, the blockade of CXCR1/2 reduced cell recruitment and the production of inflammatory mediators [85]. Blocking CXCR2 has also proven to be effective in suppressing tumor growth [104], and even in a model of lung inflammation in mice, was able to reduce the proinflammatory cytokines and several features of lung inflammation [105].

Pain caused by inflammation in the joints in patients with RA is characterized by hyperalgesia. This means that when the joint is moving in its working range and gentle pressure its applied to it, it causes stronger pain than normal [106, 107]. Pain in RA is caused by the activation of sensory nervous fibers in the inflamed synovial tissue. The hipersensibility to the pain is a normal response to inflammation [108]. However, hypersensisbility to pain can persist after resolution of the inflammatory response, and become chronic pain was studied in RA were it was reported that it persisted after the diminishment of the inflammatory parameters evaluated [109, 110]. Neutrophils infiltrated into the joint of RA patients are responsible for the secretion of several mediators that can affect, directly or indirectly pain [111]. Blocking CXCR1/2 has proven to be a valid strategy to reduce hypernocicieption in

other experimental models of Arthritis and in our work the presence and blocking of neutrophils through targeting CXCR1/2 has direct correlation with hypernociception [36, 112, 113].

Besides being an effective strategy for reducing tissue damage, blocking CXCR2 in different mice models of arthritis has proven to be effective to reduce pain. In a mice model of collagen induced arthritis, blocking CXCR2 was an effective strategy for the decrease in hypernociception measurements, inflammation and bone and cartilage degradation based on histopathology [114]. In an acute model of Septic Arthritis, blocking CXCR1/2 diminished the recruitment of neutrophils to the joint but didn't have a protective effect against joint damage [36]. In an acute AIA model, blocking CXCR1/2 did reduce tissue damage. However, treatment was administered before the intrarticular challenge, and therefore interfered on the arrival of neutrophils and the onset of inflammation [34].

In our experiments, after articular challenge the mice presented hypernociception that peaked at the 5th day after challenge but even after it declined, it didn't resolve throughout the 15 day kinetic. When prolonged AIA mice were treated with Reparixin from the 5th day forward, mechanic hypernociception resolved completely by the 12th day. This suggests that articular hypernociception in our model seems to be dependent of CXCR1/2 and neutrophils.

7. SUMMARY

1. The prolonged inflammatory model of AIA is useful to recreate many characteristics of different processes observed in real-life inflammatory diseases like RA.

2. Neutrophils are recruited to the articular cavity, the lymph node and the spleen after the induction of prolonged inflammatory AIA model.

3. Neutrophils increase their expression of MHCII and CD86 on their surface in the peak of inflammation, in the prolonged inflammatory AIA model.

4. Treatment with an antagonist of CXCR1/2 receptors causes decrease of neutrophils recruitment towards the articular cavity and the popliteal lymph node.

5. Treatment with an antagonist of CXCR1/2 receptors decreases the intensity of the expression of MHCII in neutrophils on day 10 after challenge.

6. Blockade of PMN recruitment decreases the hypernociceptive response.

7. Blockade of PMN recruitment and activation is associated with decreased tissue damage and dysfunction.

8. Blockade of CXCR1/2 with Reparixin decreased lymphocyte activation, as seen by decreased production of IFN-g in vivo and IL-17 production by splenocytes.

8. CONCLUSIONS

CXCR1/2 blockade was effective to reduce tissue inflammation, damage and hypernociception. Its effect seems to be dependent on decrease neutrophil migration and activation, as decreasing APC-like phenotype in neutrophils. Thus, CXCR1/2 could have multifactorial effects on joint pathology, ranging from innate to adaptive immune response and contribution to neuron sensitization in the model of arthritis used in this study.

9. REFERENCES

1. Kotas, M.E. and R. Medzhitov, *Homeostasis, inflammation, and disease susceptibility.* Cell, 2015. **160**(5): p. 816-827.
2. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.
3. Fullerton, J.N. and D.W. Gilroy, *Resolution of inflammation: a new therapeutic frontier.* Nat Rev Drug Discov, 2016. **15**(8): p. 551-67.
4. Nathan, C. and A. Ding, *Nonresolving inflammation.* Cell, 2010. **140**(6): p. 871-82.
5. Lo, D., et al., *Integrating innate and adaptive immunity in the whole animal.* Immunol Rev, 1999. **169**: p. 225-39.
6. McInnes, I.B. and G. Schett, *The pathogenesis of rheumatoid arthritis.* N Engl J Med, 2011. **365**(23): p. 2205-19.
7. Castaneda, S., M.T. Nurmohamed, and M.A. Gonzalez-Gay, *Cardiovascular disease in inflammatory rheumatic diseases.* Best Pract Res Clin Rheumatol, 2016. **30**(5): p. 851-869.
8. Crowson, C.S., et al., *Rheumatoid arthritis and cardiovascular disease.* Am Heart J, 2013. **166**(4): p. 622-628.e1.
9. Wright, H.L., R.J. Moots, and S.W. Edwards, *The multifactorial role of neutrophils in rheumatoid arthritis.* Nat Rev Rheumatol, 2014. **10**(10): p. 593-601.
10. Kaminsky, Z.A., et al., *DNA methylation profiles in monozygotic and dizygotic twins.* Nat Genet, 2009. **41**(2): p. 240-5.
11. Klein, K. and S. Gay, *Epigenetics in rheumatoid arthritis.* Curr Opin Rheumatol, 2015. **27**(1): p. 76-82.
12. Wegner, N., et al., *Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis.* Arthritis Rheum, 2010. **62**(9): p. 2662-72.
13. Okada, Y., et al., *Genetics of rheumatoid arthritis contributes to biology and drug discovery.* Nature, 2014. **506**(7488): p. 376-81.
14. Viatte, S., et al., *Association of HLA-DRB1 haplotypes with rheumatoid arthritis severity, mortality, and treatment response.* Jama, 2015. **313**(16): p. 1645-56.
15. Viatte, S. and A. Barton, *Genetics of rheumatoid arthritis susceptibility, severity, and treatment response.* Semin Immunopathol, 2017. **39**(4): p. 395-408.
16. Derksen, V., T.W.J. Huizinga, and D. van der Woude, *The role of autoantibodies in the pathophysiology of rheumatoid arthritis.* Semin Immunopathol, 2017. **39**(4): p. 437-446.
17. Mellado, M., et al., *T Cell Migration in Rheumatoid Arthritis.* Front Immunol, 2015. **6**: p. 384.
18. Bustamante, M.F., et al., *Fibroblast-like synoviocyte metabolism in the pathogenesis of rheumatoid arthritis.* Arthritis Res Ther, 2017. **19**(1): p. 110.
19. Alessandri, A.L., et al., *Resolution of inflammation: mechanisms and opportunity for drug development.* Pharmacol Ther, 2013. **139**(2): p. 189-212.
20. Woodrick, R.S. and E.M. Ruderman, *Safety of biologic therapy in rheumatoid arthritis.* Nat Rev Rheumatol, 2011. **7**(11): p. 639-52.

21. Tanushree Roy, S.G., *Animal models of rheumatoid arthritis: correlation and usefulness with human rheumatoid arthritis.* Indo American Journal of Pharm Research, 2013.
22. Brackertz, D., et al., *Studies on antigen-induced arthritis in mice. II. Immunologic correlates of arthritis susceptibility in mice.* J Immunol, 1977. **118**(5): p. 1639-44.
23. Crossley, M.J., M. Spowage, and I.M. Hunneyball, *Studies on the effects of pharmacological agents on antigen-induced arthritis in BALB/c mice.* Drugs Exp Clin Res, 1987. **13**(5): p. 273-7.
24. Wong, P.K., et al., *Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis.* Arthritis Rheum, 2006. **54**(1): p. 158-68.
25. Boe, A., et al., *Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis.* Cytokine, 1999. **11**(12): p. 1057-64.
26. Santos, L.L., et al., *Reduced arthritis in MIF deficient mice is associated with reduced T cell activation: down-regulation of ERK MAP kinase phosphorylation.* Clin Exp Immunol, 2008. **152**(2): p. 372-80.
27. Nissler, K., et al., *Anti-CD4 monoclonal antibody treatment in acute and early chronic antigen induced arthritis: influence on macrophage activation.* Ann Rheum Dis, 2004. **63**(11): p. 1470-7.
28. Grespan, R., et al., *CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of neutrophils to inflamed joints in mice with antigen-induced arthritis.* Arthritis Rheum, 2008. **58**(7): p. 2030-40.
29. Queiroz-Junior, C.M., et al., *Experimental arthritis triggers periodontal disease in mice: involvement of TNF-alpha and the oral Microbiota.* J Immunol, 2011. **187**(7): p. 3821-30.
30. Sousa, L.F., et al., *Blockade of CXCR1/2 chemokine receptors protects against brain damage in ischemic stroke in mice.* Clinics (Sao Paulo), 2013. **68**(3): p. 391-4.
31. Wengner, A.M., et al., *CXCR5- and CCR7-dependent lymphoid neogenesis in a murine model of chronic antigen-induced arthritis.* Arthritis Rheum, 2007. **56**(10): p. 3271-83.
32. Tanaka, D., et al., *Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis.* Immunology, 2006. **119**(2): p. 195-202.
33. Wipke, B.T. and P.M. Allen, *Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis.* J Immunol, 2001. **167**(3): p. 1601-8.
34. Coelho, F.M., et al., *The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature.* Arthritis Rheum, 2008. **58**(8): p. 2329-37.
35. Sachs, D., et al., *Cooperative role of tumour necrosis factor-alpha, interleukin-1beta and neutrophils in a novel behavioural model that concomitantly demonstrates articular inflammation and hypernociception in mice.* Br J Pharmacol, 2011. **162**(1): p. 72-83.

36. Boff, D., et al., *CXCR2 is critical for bacterial control and development of joint damage and pain in Staphylococcus aureus-induced septic arthritis in mouse.* Eur J Immunol, 2018. **48**(3): p. 454-463.
37. Nathan, C., *Neutrophils and immunity: challenges and opportunities.* Nat Rev Immunol, 2006. **6**(3): p. 173-82.
38. Nathan, C., *Points of control in inflammation.* Nature, 2002. **420**(6917): p. 846-52.
39. de Chaisemartin, L., G. Hayem, and S. Chollet-Martin, *When neutrophils cast their nets.* Joint Bone Spine, 2013. **80**(2): p. 124-6.
40. Brinkmann, V. and A. Zychlinsky, *Neutrophil extracellular traps: is immunity the second function of chromatin?* J Cell Biol, 2012. **198**(5): p. 773-83.
41. Pratesi, F., et al., *Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps.* Ann Rheum Dis, 2014. **73**(7): p. 1414-22.
42. Khandpur, R., et al., *NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.* Sci Transl Med, 2013. **5**(178): p. 178ra40.
43. Schauer, C., et al., *Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines.* Nat Med, 2014. **20**(5): p. 511-7.
44. Rollet-Labelle, E., et al., *Cross-linking of IgGs bound on circulating neutrophils leads to an activation of endothelial cells: possible role of rheumatoid factors in rheumatoid arthritis-associated vascular dysfunction.* J Inflamm (Lond), 2013. **10**(1): p. 27.
45. Kundu, S., et al., *Oxidative stress as a potential biomarker for determining disease activity in patients with rheumatoid arthritis.* Free Radic Res, 2012. **46**(12): p. 1482-9.
46. Gardi, C., et al., *Quercetin reduced inflammation and increased antioxidant defense in rat adjuvant arthritis.* Arch Biochem Biophys, 2015. **583**: p. 150-7.
47. Moon, D.O., et al., *Curcumin attenuates inflammatory response in IL-1beta-induced human synovial fibroblasts and collagen-induced arthritis in mouse model.* Int Immunopharmacol, 2010. **10**(5): p. 605-10.
48. Takashima, A. and Y. Yao, *Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell.* J Leukoc Biol, 2015. **98**(4): p. 489-96.
49. Blum, J.S., P.A. Wearsch, and P. Cresswell, *Pathways of antigen processing.* Annu Rev Immunol, 2013. **31**: p. 443-73.
50. Cross, A., et al., *Synovial fluid neutrophils transcribe and express class II major histocompatibility complex molecules in rheumatoid arthritis.* Arthritis Rheum, 2003. **48**(10): p. 2796-806.
51. Ostanin, D.V., et al., *Acquisition of antigen-presenting functions by neutrophils isolated from mice with chronic colitis.* J Immunol, 2012. **188**(3): p. 1491-502.
52. Vono, M., et al., *Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo.* Blood, 2017. **129**(14): p. 1991-2001.
53. Abi Abdallah, D.S., et al., *Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation.* Int Immunol, 2011. **23**(5): p. 317-26.

54. Hufford, M.M., et al., *Influenza-infected neutrophils within the infected lungs act as antigen presenting cells for anti-viral CD8(+) T cells.* PLoS One, 2012. **7**(10): p. e46581.
55. Beauvillain, C., et al., *CCR7 is involved in the migration of neutrophils to lymph nodes.* Blood, 2011. **117**(4): p. 1196-204.
56. Scapini, P. and M.A. Cassatella, *Social networking of human neutrophils within the immune system.* Blood, 2014. **124**(5): p. 710-9.
57. Riise, R.E., et al., *TLR-Stimulated Neutrophils Instruct NK Cells To Trigger Dendritic Cell Maturation and Promote Adaptive T Cell Responses.* J Immunol, 2015. **195**(3): p. 1121-8.
58. Lim, K., et al., *Neutrophil trails guide influenza-specific CD8(+) T cells in the airways.* Science, 2015. **349**(6252): p. aaa4352.
59. Buckley, C.D., D.W. Gilroy, and C.N. Serhan, *Proresolving lipid mediators and mechanisms in the resolution of acute inflammation.* Immunity, 2014. **40**(3): p. 315-27.
60. Lopes, F., et al., *Resolution of neutrophilic inflammation by H2O2 in antigen-induced arthritis.* Arthritis Rheum, 2011. **63**(9): p. 2651-60.
61. Scannell, M. and P. Maderna, *Lipoxins and annexin-1: resolution of inflammation and regulation of phagocytosis of apoptotic cells.* ScientificWorldJournal, 2006. **6**: p. 1555-73.
62. Jones, H.R., et al., *The role of neutrophils in inflammation resolution.* Semin Immunol, 2016. **28**(2): p. 137-45.
63. Fattori, V., F.A. Amaral, and W.A. Verri, Jr., *Neutrophils and arthritis: Role in disease and pharmacological perspectives.* Pharmacol Res, 2016. **112**: p. 84-98.
64. Duffin, R., et al., *Targeting granulocyte apoptosis: mechanisms, models, and therapies.* Immunol Rev, 2010. **236**: p. 28-40.
65. Hallett, J.M., et al., *Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation.* Trends Pharmacol Sci, 2008. **29**(5): p. 250-7.
66. Rosas, B.C., GraçasHenriques, *Neutrophils in Rheumatoid Arthritis: A Target for Discovering New Therapies Based on Natural Products.* 2017.
67. Luster, A.D., *Chemokines--chemotactic cytokines that mediate inflammation.* N Engl J Med, 1998. **338**(7): p. 436-45.
68. Mackay, C.R., *Chemokines: immunology's high impact factors.* Nat Immunol, 2001. **2**(2): p. 95-101.
69. Massara, M., et al., *Atypical chemokine receptors in cancer: friends or foes?* J Leukoc Biol, 2016. **99**(6): p. 927-33.
70. Bonecchi, R. and G.J. Graham, *Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response.* Front Immunol, 2016. **7**: p. 224.
71. Bachelerie, F., et al., *International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors.* Pharmacol Rev, 2014. **66**(1): p. 1-79.
72. Zlotnik, A. and O. Yoshie, *Chemokines: a new classification system and their role in immunity.* Immunity, 2000. **12**(2): p. 121-7.

73. Hughes, C.E. and R.J.B. Nibbs, *A guide to chemokines and their receptors.* Febs j, 2018.
74. Chintakuntlawar, A.V. and J. Chodosh, *Chemokine CXCL1/KC and its receptor CXCR2 are responsible for neutrophil chemotaxis in adenoviral keratitis.* J Interferon Cytokine Res, 2009. **29**(10): p. 657-66.
75. Russo, R.C., et al., *The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases.* Expert Rev Clin Immunol, 2014. **10**(5): p. 593-619.
76. Bozic, C.R., et al., *The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization.* J Biol Chem, 1994. **269**(47): p. 29355-8.
77. Yoshimura, T., et al., *Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9233-7.
78. Wolf, M., et al., *Granulocyte chemotactic protein 2 acts via both IL-8 receptors, CXCR1 and CXCR2.* Eur J Immunol, 1998. **28**(1): p. 164-70.
79. Strieter, R.M., et al., *The role of CXC chemokines as regulators of angiogenesis.* Shock, 1995. **4**(3): p. 155-60.
80. Talbot, J., et al., *CCR2 Expression in Neutrophils Plays a Critical Role in Their Migration Into the Joints in Rheumatoid Arthritis.* Arthritis Rheumatol, 2015. **67**(7): p. 1751-9.
81. Bizzarri, C., et al., *ELR+ CXC chemokines and their receptors (CXC chemokine receptor 1 and CXC chemokine receptor 2) as new therapeutic targets.* Pharmacol Ther, 2006. **112**(1): p. 139-49.
82. Gorio, A., et al., *Reparixin, an inhibitor of CXCR2 function, attenuates inflammatory responses and promotes recovery of function after traumatic lesion to the spinal cord.* J Pharmacol Exp Ther, 2007. **322**(3): p. 973-81.
83. Barcelos, L.S., et al., *Impaired inflammatory angiogenesis, but not leukocyte influx, in mice lacking TNFR1.* J Leukoc Biol, 2005. **78**(2): p. 352-8.
84. Alam, J., I. Jantan, and S.N.A. Bukhari, *Rheumatoid arthritis: Recent advances on its etiology, role of cytokines and pharmacotherapy.* Biomed Pharmacother, 2017. **92**: p. 615-633.
85. Khanam, A., et al., *Blockade of Neutrophil's Chemokine Receptors CXCR1/2 Abrogate Liver Damage in Acute-on-Chronic Liver Failure.* Front Immunol, 2017. **8**: p. 464.
86. Jaillon, S., et al., *Neutrophils in innate and adaptive immunity.* Semin Immunopathol, 2013. **35**(4): p. 377-94.
87. Klareskog, L., A.I. Catrina, and S. Paget, *Rheumatoid arthritis.* Lancet, 2009. **373**(9664): p. 659-72.
88. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
89. Oliveira, M.C., et al., *Tumor Necrosis Factor, but Not Neutrophils, Alters the Metabolic Profile in Acute Experimental Arthritis.* PLoS One, 2016. **11**(1): p. e0146403.
90. Daeron, M., et al., *Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future.* Immunol Rev, 2008. **224**: p. 11-43.

91. Kirkham, B.W., et al., *Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort).* Arthritis Rheum, 2006. **54**(4): p. 1122-31.
92. Mateen, S., et al., *Understanding the role of cytokines in the pathogenesis of rheumatoid arthritis.* Clin Chim Acta, 2016. **455**: p. 161-71.
93. van Roon, J.A., F.P. Lafeber, and J.W. Bijlsma, *Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis.* Arthritis Rheum, 2001. **44**(1): p. 3-12.
94. Kim, G.W., et al., *IL-6 inhibitors for treatment of rheumatoid arthritis: past, present, and future.* Arch Pharm Res, 2015. **38**(5): p. 575-84.
95. Scheller, J., et al., *The pro- and anti-inflammatory properties of the cytokine interleukin-6.* Biochim Biophys Acta, 2011. **1813**(5): p. 878-88.
96. Sarkar, S., et al., *Regulation of pathogenic IL-17 responses in collagen-induced arthritis: roles of endogenous interferon-gamma and IL-4.* Arthritis Res Ther, 2009. **11**(5): p. R158.
97. Kalyan, S. and D. Kabelitz, *When neutrophils meet T cells: beginnings of a tumultuous relationship with underappreciated potential.* Eur J Immunol, 2014. **44**(3): p. 627-33.
98. Leliefeld, P.H., L. Koenderman, and J. Pillay, *How Neutrophils Shape Adaptive Immune Responses.* Front Immunol, 2015. **6**: p. 471.
99. Beauvillain, C., et al., *Neutrophils efficiently cross-prime naive T cells in vivo.* Blood, 2007. **110**(8): p. 2965-73.
100. Duffy, D., et al., *Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells.* Immunity, 2012. **37**(5): p. 917-29.
101. Tarner, I.H., et al., *The different stages of synovitis: acute vs chronic, early vs late and non-erosive vs erosive.* Best Pract Res Clin Rheumatol, 2005. **19**(1): p. 19-35.
102. Elshabrawy, H.A., et al., *The pathogenic role of angiogenesis in rheumatoid arthritis.* Angiogenesis, 2015. **18**(4): p. 433-48.
103. Erdem, H., et al., *Synovial angiostatic non-ELR CXC chemokines in inflammatory arthritides: does CXCL4 designate chronicity of synovitis?* Rheumatol Int, 2007. **27**(10): p. 969-73.
104. Sueoka, H., et al., *Blockage of CXCR2 suppresses tumor growth of intrahepatic cholangiocellular carcinoma.* Surgery, 2014. **155**(4): p. 640-9.
105. Schneberger, D., et al., *CXCR1/CXCR2 antagonist CXCL8(3-74)K11R/G31P blocks lung inflammation in swine barn dust-instilled mice.* Pulm Pharmacol Ther, 2015. **31**: p. 55-62.
106. Sarzi-Puttini, P., et al., *Pain in rheumatoid arthritis: a critical review.* Reumatismo, 2014. **66**(1): p. 18-27.
107. Schaible, H.G., A. Ebersberger, and G.S. Von Banchet, *Mechanisms of pain in arthritis.* Ann N Y Acad Sci, 2002. **966**: p. 343-54.
108. Lee, Y.C., *Effect and treatment of chronic pain in inflammatory arthritis.* Curr Rheumatol Rep, 2013. **15**(1): p. 300.

109. Rifbjerg-Madsen, S., et al., *Pain and pain mechanisms in patients with inflammatory arthritis: A Danish nationwide cross-sectional DANBIO registry survey.* PLoS One, 2017. **12**(7): p. e0180014.
110. Lee, Y.C., et al., *Pain persists in DAS28 rheumatoid arthritis remission but not in ACR/EULAR remission: a longitudinal observational study.* Arthritis Res Ther, 2011. **13**(3): p. R83.
111. Cunha, T.M., et al., *Crucial role of neutrophils in the development of mechanical inflammatory hypernociception.* J Leukoc Biol, 2008. **83**(4): p. 824-32.
112. Barsante, M.M., et al., *Blockade of the chemokine receptor CXCR2 ameliorates adjuvant-induced arthritis in rats.* Br J Pharmacol, 2008. **153**(5): p. 992-1002.
113. Manjavachi, M.N., et al., *Spinal blockage of CXCL1 and its receptor CXCR2 inhibits paclitaxel-induced peripheral neuropathy in mice.* Neuropharmacology, 2019. **151**: p. 136-143.
114. Min, S.H., et al., *Pharmacological targeting reveals distinct roles for CXCR2/CXCR1 and CCR2 in a mouse model of arthritis.* Biochem Biophys Res Commun, 2010. **391**(1): p. 1080-6.