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ANTIBODY- AND OSTEOCLAST-DEPENDENT MECHANISMS OF CHRONIC PAIN

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Antibody- and osteoclast-dependent mechanisms of chronic pain

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To my Parents

"It does not matter how slowly you go as long as you do not stop"

— Confucius —

POPULAR SCIENCE SUMMARY OF THE THESIS

The function of the immune system is to protect us from disease and infection by producing antibodies, which neutralize pathogens. However, if it malfunctions, it mistakenly produces antibodies against our own body. This process is called autoimmune disease and it can affect cells, tissues or whole organs. Autoimmune diseases are often accompanied by pain, which is the major cause of suffering for the patients. For many years, it has been thought that pain is a result of the inflammatory process that arises in response to injury. Following that reasoning, it was thought that reducing inflammation would eliminate pain.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by swelling, redness and stiffness of the joints as well as bone loss. Despite responding well to anti-inflammatory drugs, large proportion of patients still report to have pain. Interestingly, many RA patients suffer from joint pain for a very long time before their joints become inflamed. Extensive research has shown that antibodies that circulate in the blood of RA patients can cause pain in the absence of inflammation. By using laboratory mice and exposing them to antibodies from RA patients, this thesis explores the mechanisms that can explain how antibodies cause pain, with the hope of creating a basis for improving pain treatment in the future.

Firstly, this thesis demonstrates that RA antibodies can cause pain by activating bone cells, called osteoclasts, whose main function is to break down bone. RA antibodies instruct osteoclasts to multiply in an uncontrolled manner and digest more bone than necessary. Intriguingly, not only do osteoclasts make bones weaker, but they can also release substances that cause pain. The work carried out in this thesis has led to the identification of several pain-inducing molecules which might be released from activated osteoclasts, or other cells in the joints. Importantly, while common painkillers (like naproxen or paracetamol) did not reverse the pain in mice, blocking osteoclast activity successfully blocked the pain induced by RA antibodies.

Secondly, the work performed in this thesis has also shown that RA antibodies can cause pain without causing bone destruction. By directly activating receptors located on the nerve endings in the joints, RA antibodies can trigger persistent pain without causing inflammation. Interestingly, these antibodies can also enhance the pain sensation by binding to and activating the cells that surround neurons.

Lastly, the work presented in the fourth paper of this thesis proposes that fibromyalgia syndrome (FMS) might be an autoimmune disease. Fibromyalgia is a condition characterized

by chronic widespread pain in the joints and muscles, which is accompanied by fatigue and sleep disturbances. At the moment, there is no cure for fibromyalgia and the pain treatment is not effective. By exposing mice to antibodies extracted from the blood of FMS patients, we observed that they developed symptoms that are characteristic of the disease: they became highly sensitive to pressure and cold, had muscle weakness, and their overall activity diminished. Although more research is needed to fully understand how antibodies cause pain in FMS, these results suggests that therapies that modulate immune system and reduce antibody production might be of benefit for FMS patients.

In summary, this thesis explores the different mechanisms through which antibodies can cause pain in diseases like RA or FMS, with the goal to prevent the action of these antibodies and, as a result, reduce the pain that patients experience.

STRESZCZENIE POPULARNONAUKOWE ROZPRAWY DOKTORSKIEJ

Funkcją układu odpornościowego (immunologicznego) jest obrona przed infekcjami i chorobami. W tym celu, układ immunologiczny produkuje przeciwciała, które neutralizują patogeny, lecz nie niszczą komórek organizmu. Choroba autoimmunologiczna występuje w przypadku, gdy system odpornościowy samoistnie zaczyna atakować i niszczyć własne tkanki.

Jednym z objawów chorób autoimmunologicznych jest przewlekły ból. Przez długi czas uważano, że ból wynika ze stanu zapalnego i wystarczy zahamować proces zapalny by ból ustąpił. Jednak wielu pacjentów z chorobami autoimmunologicznymi odczuwa ból nawet gdy ich choroba jest pod kontrolą farmakologiczną. W przypadku reumatoidalnego zapalenia stawów (RZS), choroby charakteryzującej się zaczerwienieniem, opuchlizną i sztywnością stawów oraz nadmierną utratą masy kostnej, pacjenci nadal skarżą się na ból w stawach, pomimo skutecznego wyleczenia stanu zapalnego. Co ciekawe, wielu pacjentów odczuwa ból na wiele miesięcy lub lat przed pojawieniem się zapalenia. Badania naukowe wykazały, że przeciwciała osób chorych na RZS mogą samoistnie powodować ból, w sposób niezależny od występowania stanu zapalnego. Celem niniejszej pracy doktorskiej było zbadanie mechanizmów, za pomocą których przeciwciała mogą powodować ból, z nadzieją na wykorzystanie tej wiedzy do stworzenia skuteczniejszych metod leczenia bólu.

W dwóch pierwszych projektach udowodniliśmy, że przeciwciała wyizolowane z krwi pacjentów chorych na RZS mogą powodować ból przez stymulację osteoklastów, komórek, których główną funkcją jest degradacja tkanki kostnej. Z użyciem modeli zwierzęcych wykazaliśmy, że przeciwciała RZS nie tylko powodują nadmierne rozpuszczanie tkanki kostnej, ale również pobudzają osteoklasty do wydzielania substancji powodujących ból. Co ważne, podawane myszom niesteroidowe leki przeciwzapalne okazały się nieskuteczne i jedynie zablokowanie aktywności osteoklastów zahamowało ból wywołany podaniem przeciwciał.

W trzecim projekcie pokazaliśmy, że przeciwciała wyizolowane od pacjentów z RZS mogą powodować ból bezpośrednio stymulując receptory znajdujące się na komórkach nerwowych oraz pośrednio stymulując komórki glejowe otaczające neurony. Terapie, które zablokują interakcje pomiędzy przeciwciałami i receptorami znajdującymi się na neuronach mogą okazać się skuteczne w leczeniu bólu przewlekłego.

W ostatnim projekcie zaproponowaliśmy, że fibromialgia ma podłoże autoimmunologiczne. Fibromialgia jest chorobą charakteryzującą się rozległym bólem mięśni, stawów oraz nadmierną wrażliwością na ucisk i niskie temperatury. Chorzy poza bólem odczuwają chroniczne zmęczenie oraz maja zaburzenia snu i nastroju. W tym projekcie wykazaliśmy, że myszy, którym podano przeciwciała od pacjentów z fibromialgią przejawiają objawy charakterystyczne dla tej choroby: są bardziej wrażliwe na zimno, ucisk, są mniej aktywne a ich siła mięśniowa jest słabsza, w porównaniu do myszy, którym podano przeciwciała od osób zdrowych. Pomimo, że dogłębne badania są niezbędne by w pełni zrozumieć mechanizm działania tych przeciwciał, wyniki te otwierają nowe możliwości terapeutyczne dla chorych na fibromialgię.

Podsumowując, badania realizowane w ramach niniejszej pracy doktorskiej wykazały szereg mechanizmów, za pomocą których przeciwciała mogą powodować ból w chorobach takich jak RZS czy fibromialgia. Poszerzenie podstawowej wiedzy na temat interakcji pomiędzy układem immunologicznym i nerwowym pozwoli w przyszłości na stworzenie bardziej efektywnych metod leczenia bólu.

ABSTRACT

Chronic pain is a frequent condition that affects approximately 20% of worldwide population with a higher prevalence among women. Due to the lack of optimal treatment, chronic pain leads to decreased life quality as well as significant physical and psychological complications. Consequently, it has become a major socio-economical problem, creating huge expenses in the form of medical care, sick leave and loss of productivity.

Recent findings in the fields of immunology and neuroscience have underlined the important effector functions of autoantibodies in the development of several chronic pain conditions. Although pain in autoimmune diseases has been historically associated with inflammation due to the antibody-induced activation of inflammatory pathways, pain in these conditions often occurs before inflammation development or is the only symptom of the disease. Increasing amount of preclinical evidence suggests that autoantibodies engage multiple mechanisms, which give rise to hypersensitivity independent of inflammation. Thus, the aim of this thesis was to explore the mechanisms behind the pronociceptive properties of autoantibodies isolated from rheumatoid arthritis (RA) and fibromyalgia patients.

In **Study I** we have used a combination of two RA-associated monoclonal antibodies (mAbs) isolated from B cells of RA patients, to study the connection between increased bone erosion and pain in RA. We found that mice injected with 1103:01B02/1325:01B09 mAbs developed long-lasting mechanical hypersensitivity and bone erosion in the absence of overt joint inflammation. Furthermore, we found that pain-like behavior induced by 1103:01B02/1325:01B09 is associated with a moderate increase in the expression of few pro-inflammatory factors in the joints and is resistant to treatment with conventional non-steroidal anti-inflammatory drugs (NSAIDs), suggesting it does not depend on classical inflammatory processes. Instead, we demonstrated that inhibiting osteoclast activity and acid-sensing ion channel 3 (ASIC3) signaling prevented the development of 1103:01B02/1325:01B09-induced hypersensitivity. We have also established that secretory phospholipase A2 (sPLA$_2$) and lysophosphatidylcholine 16:0 (LPC 16:0) are critical mediators of B02/B09-induced mechanical hypersensitivity, since treatment with sPLA$_2$ inhibitor reversed pain-like behavior and bone erosion induced by 1103:01B02/1325:01B09 mAbs. Collectively, these findings provide a novel link between bone erosion and pain, in a state of subclinical inflammation, and progress our knowledge about the mechanisms of bone-related pain in RA.

In **Study II** we have used a monoclonal anti-citrullinated protein antibody (ACPA) previously reported to stimulate osteoclasts *in vitro*, but not tested *in vivo*. We found that intravenous (i.v.) injection of 1325:04C03 IgG into mice induced pain-like behavior in the absence of visible signs of joint inflammation and in a fashion that is insensitive to conventional analgesics like NSAIDs or gabapentin. Instead, 1325:04C03-induced mechanical hypersensitivity was attenuated by nerve growth factor (NGF)-neutralizing antibody and a CXCR1/2 antagonist. Although 1325:04C03 only mildly stimulated osteoclast activity and did not lead to bone erosion *in vivo*, treatment with osteoclast inhibitor zoledronate partially reversed 1325:04C03-induced mechanical hypersensitivity, indicating a contribution of the bone compartment to pain-like behavior. Furthermore, increased expression of *Ngf* and neurotrophin 3 (*Ntf3*) in the ankle joints, as well as increased expression of several pronociceptive factors in the dorsal root ganglia (DRG), was prevented by osteoclast inhibition, pointing towards a relationship between altered bone metabolism and NGF-driven nociception. In conclusion, our data point to a concomitant role of NGF and osteoclast-derived ligands in mediating ACPA-induced pain-like behavior.

In **Study III** we have focused on the pronociceptive properties of 1325:01B09 mAb, an anti-modified protein antibody (AMPA) with reactivity towards several modified peptides. Intravenous injection of 1325:01B09 into mice induced mechanical and thermal hypersensitivity without any visual, histological or transcriptional signs of inflammation in the joint and was not alleviated by NSAID treatment. Instead, we found that 1325:01B09 caused a global increase in expression of several inflammatory-, macrophage-, satellite-glia cells (SGC)- and nociceptor-related factors in lumbar DRGs. Using transgenic mice that lack activating Fc-gamma receptors (FcγRs) we found that FcγRs are critical for the development of intravenous and intra-articular 1325:01B09-induced mechanical hypersensitivity and partially drive transcriptional changes in the DRGs. Finally, we have observed that 1325:01B09 binds SGCs *in vitro,* and in synergy with LPS, stimulates cells to release CXCL1. Overall, our findings point to the possibility that 1325:01B09 forms immune complexes and induces mechanical hypersensitivity through stimulation of FcγRs. Further studies are warranted to delineate if 1325:01B09 acts also locally in the DRGs by binding to SGC epitopes or FcγRI located on DRG macrophages.

In **Study IV** we have provided evidence for the pronociceptive properties of IgG isolated from serum of patients with fibromyalgia syndrome (FMS). We have shown that passive transfer of FMS IgG, but not IgG from healthy controls (HC) or IgG-depleted FMS serum

into mice, gave rise to pressure, mechanical and thermal hypersensitivity in mice, accompanied by decreased muscle strength and diminished locomotor activity. Moreover, injection of FMS IgG resulted in enhanced nociceptor responsiveness to mechanical and cold stimulation in the skin as well as loss of intraepidermal innervation. While FMS IgG did not directly activate sensory neurons, it bound to SGCs *in vitro* and *in vivo* and stimulated their activity measured by immunohistochemistry and gene expression. Furthermore, FMS IgG did not accumulate in mouse spinal cord or brain. Instead, FMS IgG bound to human DRGs and colocalized with both SGCs and sensory neurons. To conclude, these findings demonstrate that passive transfer of IgG from FMS patients into mice recapitulates key features of the disease. While in-depth studies are required to understand the cellular and molecular mechanisms of FMS IgG-induced hypersensitivity, we suggest that therapies that reduce IgG titres or decrease IgG binding might be successful in treating symptoms of FMS.

In summary, this thesis underlines the urgency of in-depth understanding of mechanisms behind autoantibody-induced pain in diseases like rheumatoid arthritis or fibromyalgia syndrome. Studies described here provide evidence that autoreactive antibodies engage several inflammation-independent mechanisms to induce pain and provide novel insights that can aid in designing better pain therapies in the future. Furthermore, only by undertaking translational studies like the ones described above we will be able to understand the complex mechanisms that drive pain in RA or FMS.

LIST OF SCIENTIFIC PAPERS

I. **Antibody-induced pain-like behavior and bone erosion – links to subclinical inflammation, osteoclast activity and ASIC3-dependent sensitization.** Alexandra Jurczak[*], Lauriane Delay[*], Julie Barbier, Nils Simon, Emerson Krock, Katalin Sandor, Nilesh M. Agalave, Resti Rudjito, Gustaf Wigerblad, Katarzyna Rogóż, Arnaud Briat, Elisabeth Miot-Noirault, Arisai Martinez-Martinez, Dieter Brömme, Caroline Grönwall, Vivianne Malmström, Lars Klareskog, Spiro Khoury, Thierry Ferreira, Bonnie Labrum, Emmanuel Deval, Juan Miguel Jiménez-Andrade, Fabien Marchand[#], Camilla I. Svensson[#]. *PAIN* 2021 Nov 19. Online ahead of print

II. **Contribution of NGF signaling to ACPA-induced pain-like behavior.** Alexandra Jurczak, Nils Simon, Katalin Sandor, Emerson Krock, Julie Barbier, Alex Bersellini Farinotti, Katarzyna Rogóż, Jacob B. Olesen, Arisai Martinez-Martinez, Bence Rethi, Vivianne Malmstrom, Lars Klareskog, Caroline Grönwall, Kent Sœ, Juan Miguel Jimenéz-Andrade, Fabien Marchand, Camilla I. Svensson. *Manuscript*

III. **Insights into FcγR involvement in RA-associated autoantibody-induced pain-like behavior.** Alexandra Jurczak, Emerson Krock, Katalin Sandor, Alex Bersellini Farinotti, Nilesh M. Agalave, Katarzyna Rogóż, Julie Barbier, Resti Rudjito, Arisai Martinez-Martinez, Vivianne Malmstrom, Caroline Grönwall, Lars Klareskog, Juan Miguel Jimenéz-Andrade, Fabien Marchand, Camilla I. Svensson. *Manuscript*

IV. **Passive transfer of fibromyalgia symptoms from patients to mice.** Andreas Goebel[*], Emerson Krock[*], Clive Gentry, Mathilde R. Israel, Alexandra Jurczak, Carlos Morado Urbina, Katalin Sandor, Nisha Vastani, Margot Maurer, Ulku Cuhadar, Serena Sensi, Yuki Nomura, Joana Menezes, Azar Baharpoor, Louisa Brieskorn, Angelica Sandström, Jeanette Tour, Diana Kadetoff, Lisbet Haglund, Eva Kosek, Stuart Bevan, Camilla I. Svensson[#], and David A. Andersson[#]. *J Clin Invest.* 2021 Jul 1;131(13):e144201

[*],[#] Contributed equally

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. **Tyrosine kinase type A-specific signaling pathways are critical for mechanical allodynia development and bone alterations in a mouse model of rheumatoid arthritis.** Lauriane Delay, Julie Barbier, Youssef Aissouni, <u>Alexandra Jurczak</u>, Ludivine Boudieu, Arnaud Briat, Philippe Auzeloux, Célia Barrachina, Emeric Dubois, Denis Ardid, Elisbeth Miot-Noirault, Camilla I Svensson, Aziz Moqrich , Fabien Marchand. *PAIN* 2021 Sep 23, Online ahead of print

II. **Lyso-phosphatidyl-choline 16:0 mediates persistent joint pain through Acid-Sensing Ion Channel 3: preclinical and clinical evidences.** Florian Jacquot, Spiro Khoury, Bonnie Labrum, Kevin Delance, Ludivine Pidoux, Julie Barbier, Lauriane Delay, Agathe Bayle, Youssef Aissouni, David A. Barriere, Kim Kultima, Eva Freyhult, Anders Hugo, Eva Kosek, Aisha Ahmed, <u>Alexandra Jurczak</u>, Eric Lingueglia, Camilla I. Svensson, Veronique Breuil, Thierry Ferreira, Fabien Marchand, Emmanuel Deval. *PAIN* 2022 in press

III. **Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis.** Teresa Fernandez-Zafra, Tianle Gao, <u>Alexandra Jurczak</u>, Katalin Sandor, Zoe Hore, Nilesh M. Agalave, Jie Su, Johanna Estelius, Jon Lampa, Tomas Hökfelt, Zsuzsanna Wiesenfeld-Hallin, Xiaojun Xu, Franziska Denk, Camilla I. Svensson. PAIN 2019 Jan;160(1):224-236

IV. **Pain pathogenesis in rheumatoid arthritis – what have we learned from animal models?** Emerson Krock, <u>Alexandra Jurczak,</u> Camilla I. Svensson. PAIN 2018 Sep; 159 Suppl 1:S98-S109

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody
AAPAs	Anti-acetylated protein antibodies
ACPAs	Anti-citrullinated protein antibodies
ACarPAs	Anti-carbamylated protein antibodies
AMPAs	Anti-modified protein antibodies
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CAIA	Collagen-antibody induced arthritis
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRPS	Complex regional pain syndrome
Ctsk	Cathepsin K
CXCL	Chemokine CXC motif ligand
CXCL1	Fractalkine
CXCR	CXC chemokine receptor
DMARD	Disease-modifying anti-rheumatic drug
DRG	Dorsal root ganglia
FcγR	Fc-gamma receptors
FMS	Fibromyalgia syndrome
GABA	Gamma aminobutyric acid
GFAP	Glial-fibrillary acidic protein
GPCRs	G protein-coupled receptors
GS	Glutamine synthetase
GWAS	Genome wide association studies

IL	Interleukin
i.a.	Intra-articular
i.p.	Intra-peritoneal
i.v.	Intravenous
Kir 4.1	Inward rectifying potassium channel
LPC	Lysophosphatidylcholine
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
NF200	Neurofilament 200
NGF	Nerve growth factor
NGS	Normal goat serum
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PAD	Peptidyl-arginine deiminase
PBS	Phosphate buffered saline
PTM	Post-translational modification
RA	Rheumatoid arthritis
SGC	Satellite glia cell
SLE	Systemic lupus erythematosus
SP	Substance P
sPLA$_2$	Secretory phospholipase A$_2$
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
TrkA	Tropomyosin receptor kinase A
TRPV1	Transient receptor potential vanilloid 1
TSPO	Translocator protein
V-ATPase	Vacuolar H$^+$ - ATPase a3 isoform

1 INTRODUCTION

Chronic pain is one of the leading causes of suffering and the major reason for why patients seek medical care (1,2). It is defined as pain that lasts longer than 3 months or beyond the expected period of healing (3). Although chronic pain affects approximately 10-20% of the world population (4,5), with a higher prevalence among women (6,7), it continues to be a highly under-recognized and sub-optimally treated condition. Persistent pain is associated with huge emotional distress, physical disability and depression and thus generates large socio-economical costs due to loss of productivity and reduced work effectiveness (8). Despite a growing array of treatment options, the overall effectiveness of pain therapy is poor and inconsistent, with more than 40% of patients complaining about insufficient pain relief (5). Thus, persistent pain has been recognized as not simply a chronological extension of acute pain, but disease itself, which requires novel therapeutic strategies (9,10).

Musculoskeletal disorders including rheumatoid arthritis (RA), osteoarthritis (OA), fibromyalgia syndrome (FMS) and low-back pain are the most common causes of chronic pain and physical disability, with the prevalence varying between 20-30% in different populations (11). Musculoskeletal pain affects bones, joints, muscles, ligaments and tendons and occurs in response to numerous factors like trauma, inflammation, autoimmune disease or aging. Furthermore, chronic musculoskeletal pain is rarely limited to a specific body site, but instead affects multiple sites or presents itself as widespread pain (12). While musculoskeletal pain conditions are very complex and involve multiple types of pain and multiple etiologies, they all lack therapies that satisfactorily manage pain without unwanted side effects (13,14). This is why a better understanding of mechanisms and factors driving skeletal pain is urgently needed in order to identify new strategies for pain-relief.

Although mechanistically pain arises and is transmitted in the nociceptive system, over the past two decades it has become apparent that sensitization and prolonged activity in the nociceptors can be mediated and sustained also by other cells like immune and glial cells (15,16). Growing evidence points towards a bidirectional signaling between the nociceptive and immune systems and a role of both adaptive and innate immunity in activating and sensitizing sensory fibers (17). Furthermore, it is well established that immune cells play an important role in the transition from acute to chronic pain in several painful conditions and can act at various anatomical levels: in the damaged tissue, peripheral nerves or central nervous system (CNS) (15). A wide range of immune mediators, known to enhance (or reduce) pain signaling, is

released from immune cells including cytokines, chemokines, nitric oxide, growth factors and protons. Although neutralizing the action of several of these factors was shown to be clinically successful in the treatment of some painful conditions, targeting these mediators without interfering with the fundamental roles they play in immunity remains a challenge.

Autoimmune diseases are caused by the breakdown of self-tolerance, which leads to generation of autoreactive T cell clones that recognize self-antigens and induce antibody production, by stimulating autoantigen-specific B cells (18,19). As a consequence, autoreactive lymphocytes lead to self-tissue damage, which may be localized or systemic, depending on the distribution of self-antigens. Although autoimmune diseases differ in their pathophysiology, pain appears to be a common symptom in most of these conditions (20). Historically, pain in autoimmune diseases was ascribed to localized or systemic inflammation resulting from antibody-induced complement activation or release of proinflammatory factors. However, pain in autoimmune diseases is often present before the onset of inflammation (*e.g.* in RA) or can be the only symptom of the diseases (*e.g.* in potassium channel complex autoimmunity). This suggests that disease-specific autoantibodies can employ different mechanisms that will promote chronic pain development.

This thesis explores the mechanisms through which autoantibodies derived from serum of patients with RA and FMS induce pain-like behaviors in mice. The work presented here focuses on the interaction between autoantibodies and cells located in the joint (osteoclasts) and dorsal root ganglia (satellite-glia cells, neurons) in generating mechanical hypersensitivity in the absence of overt inflammation. The aim of this work is to expand the knowledge on the role of autoantibodies in chronic pain conditions and to identify novel targets for pain relief in autoimmune diseases.

2

2 LITERATURE REVIEW

2.1 THE NEUROBIOLOGY OF PAIN
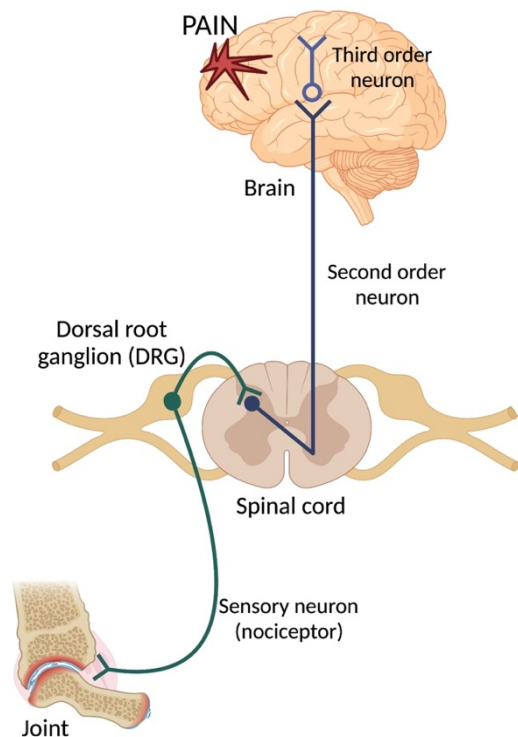
2.1.1 Pain terminology

Pain is described by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (21). Acute pain is a physiological reaction, which serves as a warning system in order to protect us from tissue damage and to elicit protective behaviors that facilitate healing. Acute pain usually lasts until nociceptive input or tissue damage have resolved, however if prolonged, continuous nociceptive stimulation can lead to the development of maladaptive chronic pain that has lost its warning signaling value (22).

Historically, pain mechanisms were classified into "nociceptive" and "neuropathic", where "nociceptive" described the "default" experience of pain induced by stimulation of primary nerve endings and "neuropathic" defined pain which resulted from a lesion or general dysfunction in the nervous system (23). This classification however was incomplete and lacked a descriptor for patients who experience pain but do not exhibit obvious signs of nociceptor activation or nerve damage, but in whom clinical assessments suggest altered nociceptive function (24–26). Examples of painful conditions that these patients suffer from include fibromyalgia, complex regional pain syndrome (CRPS), irritable bowel syndrome (IBS) and nonspecific joint pain. Thus, a third mechanistic descriptor, "nociplastic" pain, has been recently adapted. It is defined as pain that "arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain" (23,27). It is important to note that some chronic pain patients will present with both nociceptive and nociplastic pain, thus these two terms should not be regarded as exclusive categories, but rather considered as parallel in some cases (23).

2.1.2 The nociceptive system

Nociceptors are specialized sensory neurons that detect noxious (painful) stimuli and transmit them from periphery to CNS. Their cell bodies are located in the dorsal root ganglia (DRGs) from which single process emanates and bifurcates sending one axonal branch towards the periphery and one towards the dorsal horn of the spinal cord to synapse on second-order neurons. Most nociceptors are polymodal and respond to various modalities: mechanical

(painful pressure, squeezing), noxious thermal (heat and cold) and noxious chemical stimuli. Thanks to a battery of various sensor molecules in the nerve endings these different stimuli are transduced into a depolarizing sensor potential. If it is sufficiently large it opens sodium (Na^+) channels and triggers an action potential further conducted to the dorsal horn of the spinal cord or brainstem, evoking a behavioral reaction.

Figure 1. Schematic illustration showing the nociceptive pathway from ankle joint to brain. Noxious stimuli are detected by afferent nociceptors whose cell bodies are located in the dorsal root ganglion (DRG). Pseudounipolar nociceptors send signals to the dorsal horn of the spinal cord and synapse onto second order projection neurons. Next, pain-related information ascends to the hypothalamus. From there the third order neurons transmit the painful stimulus to somatosensory cortex where conscious pain perception is formed. Created with Biorender.com.

2.1.3   Nociceptor classification

Pain-sensing fibers can be broadly classified based on their diameter and degree of myelination, which determines the velocity of action potential. Anatomically, there are two major classes of nociceptive fibers: medium diameter myelinated Aδ afferents, which convey acute, well-localized, fast pain with a conduction velocity of 5-30 m/s, and unmyelinated C fibers that mediate poorly localized, slow pain at a speed of 0.4-1.4 m/s. Aδ fibers are heterogeneous and can be classified into two different types: type I fibers that respond to mechanical and chemical stimuli, but with a high heat threshold, and type II fibers with a lower heat threshold but much higher mechanical threshold compared to type I. Unmyelinated C fibers are also heterogeneous and polymodal and respond to both heat and mechanical stimulation. One subset that is thought to have an important role in chronic pain is the so-called "silent nociceptors" that are heat responsive, but mechanically insensitive in physiological states. In pathological conditions silent nociceptors become recruited to sense mechanical stimuli, but also become much more responsive to chemical stimuli like capsaicin or histamine, compared to other C fibers (28,29).

Nociceptors are excitatory neurons and release glutamate as their primary neurotransmitter. However, they can differentially express a variety of biochemical markers. For example, C-fibers can be further classified based on the content of peptides and the site within spinal cord where their central projections terminate. Peptide-rich fibers express neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP), and can be regulated by nerve

growth factor (NGF) via receptor – tyrosine receptor kinase A (TrkA). These fibers terminate predominantly in the superficial layers of the spinal dorsal horn. The "non-peptidergic" nociceptors selectively bind isolectin B4 (IB4) and express purinergic receptor P_2X_3 and receptors for glial cell line-derived neurotrophic factor (GDNF) *e.g.* tyrosine kinase c-RET (C-RET) (30). Their central projections terminate primarily within the deeper parts of lamina II of the spinal dorsal horn (31). These two groups of C fibers mediate similar types of painful stimuli and express common receptors including vanilloid receptor for capsaicin (TRPV1), which transduces both chemical and thermal stimuli (32,33). On the other hand, Aδ fibers can be characterized based on their expression of 200-kDa neurofilament (NF200), but similarly to C fibers can express CGRP and receptors for neurotrophins belonging to NGF family. Their central projections terminate primarily to layer I and the deeper layers of the dorsal horn of the spinal cord (IV and V).

2.1.4 Signal initiation and transduction

Painful stimuli are detected and processed by nociceptors thanks to various surface proteins, including ion channels, G protein-coupled receptors (GPCRs) and receptors for neurotrophins and cytokines (34). Subsequent to the detection of noxious stimuli, surface proteins initiate and propagate electrical signals towards CNS and their presence highly determines the excitability of the nociceptors and the activity in pathways in the CNS. The crucial role of ion channels/receptors is well visible in the pathophysiological states, where abnormalities in their function strongly influence and modulate the perception of pain in both animal models and humans (30).

Ion channels have received the most attention in the process of identifying the mechanisms of mechano- and thermo-transduction. Some are directly responsible for transduction (*e.g.* transient receptor potential channels, TRP) and others for transmission (*e.g.* voltage-dependent sodium channel Nav 1.8) (35). For the purpose of this thesis, ion-channels that are most relevant for the included studies will be briefly described.

The TRP receptor family is structurally diverse and detects a wide variety of stimuli. As such, they are one of the most studied groups of ion channels in the pain field. Transient receptor potential vanilloid 1 (TRPV1) is a well-characterized member of TRP receptor family, which can be activated by several stimuli including noxious heat, low pH and a wide range of endogenous and exogenous compounds including capsaicin and lipids. TRPV1 is widely expressed in both non-neuronal (skin, bladder, heart, kidney) and neuronal tissues (including sensory neurons with both C and Aδ nociceptors), where it subserves important homeostatic

functions (36). Several studies have shown its crucial role in various experimental pain states including inflammatory pain, bone-cancer and neuropathic pain (37). The analgesic potential of TRPV1 blockade has been confirmed in preclinical studies with various approaches ranging from gene disruption, receptor antagonist or neutralizing antibodies (37–40). However, clinical trials provide evidence that targeting TRPV1 in an efficacious way, without major side effects like hyperthermia, continues to be a challenge (41,42).

Several noxious processes, such as inflammation, cause a decrease in extracellular pH, which is detected by a family of proton-gated Na^+ and Ca^{2+} channels. Acid-sensing ion channels (ASICs) sense a wide pH range and are expressed in central and peripheral neurons throughout the whole pain pathway (43,44). ASIC1 and ASIC3 show the highest sensitivity to protons (H^+) and become activated by pH 7.0 and pH 7.2 respectively (45). ASIC3 generates a biphasic current with a sustained component, which is believed to be important in the non-adapting pain caused by tissue acidosis, as it does not fully inactivate and has the ability to sensitize nociceptors to other types of stimuli (46,47). Several endogenous mediators potentiate ASIC3 currents in DRG neurons including lysophosphatidylcholine (LPC) (48), arachidonic acid (AA) (49), lactate (50), serotonin (51), nitric oxide (52) and adenosine triphosphate (ATP) (53). Importantly, lipids like LPC or AA were shown to not only potentiate acid-induced ASIC3 currents but also to directly activate ASIC3, in the absence of extracellular acidification (48,54). Preclinical studies have shown that using pharmacological and genetic tools to modulate ASIC3 activity or reduce its expression in the periphery, can have an analgesic effect in several pain conditions *e.g.* arthritis, bone-cancer pain or fatigue-induced hyperalgesia (55–59). In **Study I** we have demonstrated that inhibition of ASIC3 signaling prevented the development of autoantibody-induced mechanical hypersensitivity in mice and that LPC 16:0 and secretory phospholipase A2 (sPLA$_2$, enzyme required for LPC production) contribute to sensitization in this model.

G protein-coupled receptors are the largest class of sensory proteins and are widely distributed in the peripheral and central nervous system (60). They play important roles in the regulation of nociceptors excitability, including modulating the function of ion channels (61,62) and serve as important therapeutic targets for pain management. Despite their molecular and functional diversity, all GPCRs consist of seven transmembrane domains linked by intracellular and extracellular loops. Once activated, GPCRs undergo conformational changes and promote intracellular pathways related to adenylate cyclase with the activation of downstream kinases like PKA, PKC, ERK or p38. GPCRs are activated by various ligands including neurotransmitters, ions, hormones, cytokines and chemokines as well as lipids and eicosanoids.

6

Although several GPCRs are successfully targeted for pain relief (*e.g.* opioid receptors), the function of numerous GPCRs and their ligands is still unknown (63). The variety and the complexity of GPCR family gives the possibility for identifying and characterizing potential analgesics that will treat different pain modalities with higher efficacy and less side effects (61).

Neurotrophic factors are essential for axonal growth and guidance during development (64,65) but also important in injury-associated pain and sensitivity in adulthood (66). Two main classes of trophic factors include the neurotrophin family and glial cell-line derived family. Among neurotrophins, nerve growth factor (NGF) plays a profound role in nociception, causing long-lasting sensitization of sensory neurons upon administration, in both rodents and humans (67,68). NGF exerts its effects by activating two receptors, the high affinity tropomyosin receptor kinase A (TrkA), and the low affinity neurotrophin p75 receptor. TrkA is thought to be the major mediator of NGF's pronociceptive properties (69,70). It is expressed by 80% of the nociceptors innervating skeletal structure including the joint and bone (71,72), but also by other cells in the joint such as mast cells (73), fibroblasts-like synoviocytes (FLS) (74), macrophages (75), osteoblasts (76,77) and chondrocytes (78). Upon binding to the extracellular region of TrkA located on the peripheral terminals of Aδ and C fibers, NGF is internalized into endosomes where it can be recycled, retrogradely transported to DRG or degraded (79). NGF-TrkA complex exerts short-term effects on nociception by stimulating the release of inflammatory mediators (bradykinin, histamine, ATP, prostaglandins) from *e.g.*, mast cells, basophils or macrophages, and enhancing the activity of nociceptive ion channels to promote depolarization and sensitization of the peripheral nociceptive terminals (80–82). Long-term effects of TrkA-NGF signaling occur in the DRG soma and are a result of retrograde axonal transport of the complex. Once in the DRG, NGF-TrkA complex enhances the expression and membrane localization of receptors like TRPV1 and ASIC3 as well as increases the levels of pain-modulating factors including SP, CGRP and brain-derived neurotrophic factor (BDNF). Overall, these NGF-induced cellular changes further contribute to increased sensory neuron excitability and central sensitization (82). In **Study II**, we have explored the role of NGF signaling in pain-like behavior induced by an ACPA isolated from a patient with RA. We found that neutralizing NGF reverses mechanical hypersensitivity induced by the autoantibody, but it does not reverse the transcriptional changes in the DRGs.

Although *cytokines and chemokines* were previously exclusively associated with immune cells they are now considered as general signaling molecules (83,84). Secreted from several cell types including neurons, immune and glial cells they are released both in peripheral and central nervous system, where they directly modulate the nociceptive response in a short- and long-term manner. Cytokines are further classified based on their biological function into pro- or anti-inflammatory. Examples of pro-inflammatory cytokines are interleukins (IL) 1β, 2, 6 and 7 and tumor necrosis factor-α (TNF- α) while examples of anti-inflammatory are IL-4, -10 and -13 and transforming growth factor β (TGF-β). There is abundant preclinical evidence that receptors for several cytokines and chemokines (*e.g.* IL-1β and TNF-α) are present on nociceptors and that these molecules can contribute to persistent pain states by directly activating and sensitizing nociceptors (83,85–88). Inhibition of IL-1β and TNF-α using neutralizing antibodies was shown to inhibit the development of hyperalgesia and allodynia in several pain models (89,90). Of relevance for this thesis, the chemotactic cytokine CXCL1 (murine homologue of human IL-8) can directly activate or sensitize nociceptors by binding to its receptors CXCR1 and CXCR2 present on the sensory neurons (91,92). By modulating the activity and expression of several ion channels like TRPV1 or Nav1.7, CXCL1 can cause changes in neuronal excitability that lead to peripheral sensitization (93–95). Blocking CXCL1-CXCR2 signaling was shown to be effective in treating both inflammatory and neuropathic pain in animal models (91,92), while its efficacy in humans is still not studied. In **Study II** we have shown that reparixin, a non-competitive, allosteric CXCR1/2 antagonist, prevents the development of pain-like behavior induced by a monoclonal ACPA.
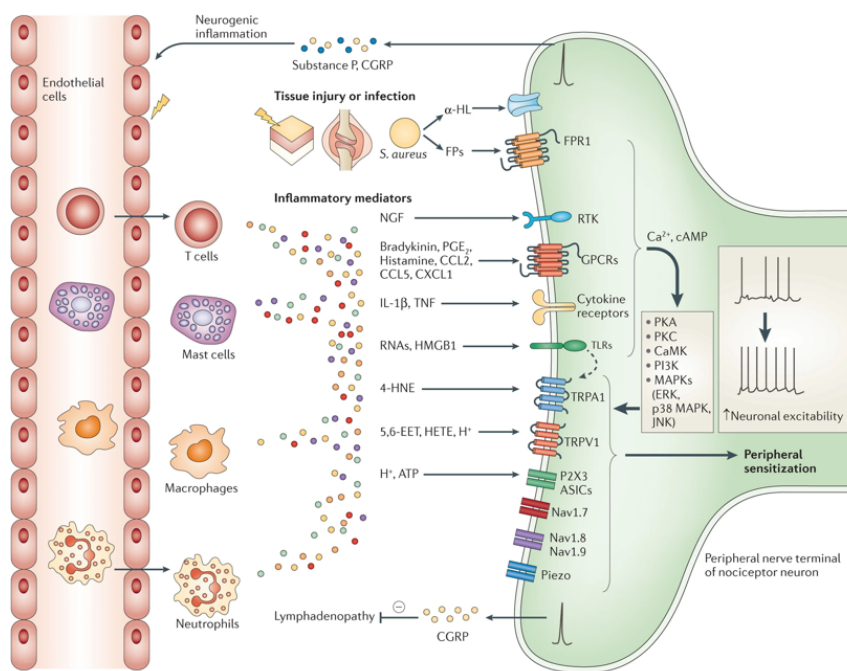

Figure 2. Nociceptor heterogeneity. Sensory neurons express a battery of surface proteins on their peripheral nerve fibers that allow them to respond to noxious stimuli. Reprinted with permission and adapted from Ji et al. 2014

2.1.5 Peripheral and central sensitization

"Sensitization" is defined as increased excitability and reduced threshold of neurons to stimulation or sensory input. Pain hypersensitivity occurs in two forms: (I) as allodynia, when non-noxious stimuli produce pain, and (II) hyperalgesia, when noxious stimuli produce an exaggerated and prolonged pain response (96). These phenomena are part of the adaptive response as they assist with the healing process by preventing or minimizing the contact with the injured tissue. However, when triggered by a disease or a maladaptive reaction to external stimuli, pain hypersensitivity can result in non-adaptive, chronic pain that outlives tissue damage or occurs without apparent cause.

Sensitization of the nociceptive system can occur both in peripheral and central nervous system and these two processes contribute to chronic pain.

Peripheral sensitization is defined as increased responsiveness of the nociceptor circuit at the level of the periphery. This often results from tissue damage and/or inflammation as well as damage to nerve fibers themselves. The major source of pain mediators, sometimes referred to as "the inflammatory soup", are immune cells like mast cells, neutrophils, macrophages, or platelets. These nociceptor-sensitizing factors include CGRP, SP, bradykinin, NGF, ATP, cytokines, prostaglandins, lipids etc. Peripheral alterations in response to these mediators include reduction in the nociceptors' excitability threshold, increase of their receptive field and recruitment of non-nociceptive neurons, which start to respond to mechanical and thermal stimuli and further amplify pain signal.

Central sensitization is defined by IASP as an "increase in nociceptor responsiveness within the central nervous system to the normal or sub threshold afferent input". It was identified as contributing factor in several chronic pain conditions (97), including: neuropathic pain (98), inflammatory pain (27,99,100), osteoarthritis (101), fibromyalgia (102) and migraine (103). Although historically central sensitization was thought to reflect the presence of peripheral noxious stimuli, it is now known to occur also in the absence of either peripheral pathology or noxious stimuli (104). Dorsal horn neurons that are subjected to central sensitization exhibit the following features: reduced threshold for activation by peripheral stimuli, development or increase in spontaneous activity, increased responses to suprathreshold stimulation and enlargement of their receptive fields (104). In addition to neuronal changes, activation of CNS glia cells (astrocytes, microglia) is thought to be the contributing factor in central sensitization. In physiological state, astrocytes and microglia surveil their environment to maintain homeostasis and proper neuronal function in the CNS. They can become activated in response

to pathological events and respond to mediators released from sensitized peripheral nociceptors. Upon pain-related activation, glia can synthesize and release several pronociceptive mediators including cytokines, chemokines and neurotrophins like TNF-α, IL1-β or BDNF, that further enhance the pain transmission via activation of key signaling pathways in nociceptive neurons (105).

2.1.6 Satellite glial cells (SGC) and pain

Satellite glial cells (SGCs) are uniquely located as they wrap around neuronal cell bodies of sensory ganglia. The distance between glial and neuronal surfaces in the DRGs is approximately 20 nm (106) and allows for bi-directional neuron-SGC interactions forming a unique structure (neuron-glial unit) not found anywhere else in the nervous system (107). Despite the close contact, SGCs do not form an absolute barrier around the sensory neurons, allowing micro- and macromolecules as well as other cells (*e.g.* macrophages, leukocytes) (108,109) to penetrate the SGC sheath. Importantly, SGCs express potassium (K+) channels as well as gamma aminobutyric acid (GABA) and glutamate transporters that allow them to control the microenvironment in sensory ganglia (107) and provide support and modulation of sensory neurons. Furthermore, as part of the SGC-neuron signaling, SGCs express receptors for several neurotransmitters and factors released from neurons, most notably ATP acting on purinergic receptors (P$_2$Rs) or cytokines (107,110).

SGCs express several proteins that allow for their identification: glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and S100 proteins. At the moment GS is considered the most useful SGC marker as GFAP exhibits low expression under naive conditions and S100 proteins can be also found in a subset of sensory neurons, Schwann cells and oligodendrocytes (111–113).

The role of SGCs in chronic pain has been studied in several preclinical models of nerve injury and inflammatory pain. Their crucial contribution to the development of chronic pain conditions is reflected by the fact that reversing injury-induced phenotypic changes in the SGCs attenuates pain-like behaviors in a number of pain models (114–117). Similarly to other glial cells, SGCs respond to nerve injury or inflammation by changing their morphology (118) and proliferating (119,120). Upregulation of GFAP in SGCs was also described in response to inflammation (121–123), but not in mouse nerve injury models (124). In addition to increased number of cells, an increased coupling between SGCs was reported in models of inflammatory pain (122,125), axotomy (117) and chemotherapy-induced neuropathy (116). This phenomenon results from increased expression of gap junction proteins (*e.g.* connexin 43)

between the cells and contributes to the spread and enhancement of neuronal excitation (126). Based on these findings, blocking gap junctions was suggested as an effective novel target for analgesic drugs (126). Furthermore, it was shown that peripheral inflammation and nerve injury suppress the function of the inward rectifying K^+ channel in SGCs (Kir4.1) (127,128), which in turn leads to SGC depolarization and release of factors like ATP or glutamate that activate sensory neurons (129). In addition, knockdown of Kir4.1 expression in trigeminal ganglia was shown to induce spontaneous pain-like behaviors in uninjured rats (127), demonstrating the importance of K^+ buffering for the development of chronic pain. In addition to small neurotransmitter molecules like ATP, reactive SGCs use cytokines to modify neuronal activity in pathological conditions. Expression of proinflammatory cytokines and chemokines like TNF-α, IL1-β, IL-6 or CX3CL1 (fractalkine) and their receptors was shown to be increased in SGCs in response to various insults (130–133). CGRP released from activated neurons was shown to further stimulate the release of cytokines from SGCs, sustaining the cross-excitation between neurons and SGCs (133–135).

2.1.7 Bone pain

2.1.7.1 Osteoclasts and pain

Pain arising from the skeletal structures, often referred to as bone pain, is a symptom of several musculoskeletal disease including RA, OA, bone cancer, Paget's disease and osteoporosis. Although these conditions differ in their pathophysiology, they often share the features of increased bone resorption and structural changes in bone microarchitecture. Studies in the field of skeletal pain provide increasing evidence for the role of osteoclasts, cells that degrade bone, in eliciting hypersensitivity. In fact, osteoclasts were shown to be increased and hyperactivated in animal models of different bone pathologies (59,136–140). Moreover, preclinical data indicate that osteoclast inhibitors like bisphosphonates (136,139–141), calcitonin (142) or osteoprotegerin (143) have anti-nociceptive properties. Also, some human studies show that antiresorptive agents are associated with pain relief (144–147). Intriguingly, osteoclast inhibitors, predominantly bisphosphonates, were demonstrated to have analgesic effects even in conditions in which osteoclasts are not primarily involved, such as inflammatory or neuropathic pain (148–150). This paradox suggests that osteoclasts produce pronociceptive factors independent of their erosive activity or that antiresorptive agents exert their anti-nociceptive effects through alternative mechanisms of action. In **Study II** we have demonstrated that pain-like behavior induced by a single monoclonal ACPA, which stimulates osteoclast differentiation *in vitro*, but does not induce bone erosion *in vivo*, is reversed by

treatment with a bisphosphonate, zoledronate. Our findings point to a possible disconnection between erosive and pronociceptive action of osteoclasts in this model.

Osteoclasts are myeloid-derived, large multinucleated cells, that reside on the surface of bones where they are responsible for bone degradation, which is an important part of homeostatic bone turnover in physiological conditions. Their differentiation is tightly controlled by macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-β ligand (RANKL) produced by osteoblasts (bone-forming cells) and surrounding stromal cells (151). In order to resorb bone, osteoclasts promote acidic and lytic milieu by secreting protons (H+) through vacuolar H+-ATPase (V-ATPase), which together with proteolytic enzymes like cathepsin K allow for bone matrix breakdown (152). While low pH is required for optimal bone resorption, protons are known to be potent pain-inducing mediators via activating proton-sensing ion channels located on the nociceptors (30). Two main classes of cation channels: ASIC3 and TRPV1 are expressed on sensory fibers innervating the bone. It has been shown that inhibition of these channels attenuates pain behaviors in several animal models of bone pain (58,153–155), pointing to an important role of osteoclast-mediated acidification in pain signal transduction.

Recent findings provide evidence that besides protons, osteoclasts produce and release also other factors known to modulate pain signaling. Among pronociceptive agents, osteoclasts were shown to release various cytokines (*e.g.* TNF-α, IL-1α, IL-6, IL-8), which by binding to their receptors located on sensory neurons are capable of eliciting nociception (138,156–158). Furthermore, osteoclasts were shown to produce and release complement fragment 3a (159), which can indirectly contribute to pain sensation by activating resident inflammatory cells (*e.g.* mast cells) or attracting other immune cells (e.g. neutrophils), which further produce algogenic substances like histamine, NGF and others (160). Moreover, several lines of evidence suggest that osteoclasts are capable of producing and metabolizing lipids, which are essential for proper bone remodeling, but can act pronociceptive by modulating ASIC3 or TRP ion channels on sensory neurons (48,161). Expression of sPLA2, enzyme required for production of AA and LPC, was detected in human osteoclasts *in vitro* as well as in human pagetic and fetal, but not healthy, mature bones *in situ* (162). Of note, sPLA2 injection is known to induce hypersensitivity (163,164) and several studies indicate that it has a role in different pain models (165–168). Finally, osteoclasts were shown to release axonal guidance molecules like semaphorin 4D and netrin-1, known to regulate bone remodeling, and recent studies point to a role of netrin-1 in mediating bone innervation and hypersensitivity in animal models of low back pain and OA (169,170). Although a vast amount of data points towards the contribution

of osteoclasts to nociception, local or systemic administration of RANKL was not sufficient to induce pain-like behaviors in mice (171). This suggests that physiologic osteoclasts are unable to induce pain and that additional disease-specific mechanisms are needed to produce and maintain bone pain.

2.1.7.2 Bone and synovial joint innervation

Different joint structures such as synovium, periosteum, articular capsule, ligaments and surrounding joint tissues are innervated by nociceptive primary afferents and sympathetic fibers (172). While Aβ fibers can be found mainly in ligaments and fibrous capsule, Aδ and C fibers innervate most of the joint structures including meniscus, periosteum, synovium, bone marrow as well as cortical and trabecular bone. The majority of sensory fibers innervating these structures can be classified as "silent nociceptors", which become active and send nociceptive information to the CNS only following injury or inflammation (173,174). Unlike other joint tissue, healthy cartilage does not contain any nerve fibers or blood vessels (175). In contrast to skin, which is innervated by a rich variety of sensory neurons, including TrkA$^-$ and TrkA$^+$, bone and articular joint structures are innervated predominantly by TrkA$^+$ Aδ and C fibers (176). This knowledge has brought some understanding into the clinical and pre-clinical observations that anti-NGF therapy provides successful analgesia in several skeletal pain conditions including bone cancer pain and low back pain (176–178), but does not relieve chronic pain in the skin (179).

Bone and joint tissues are also richly innervated by sympathetic fibers. Although tyrosine hydroxylase (TH) positive nerve fibers are mainly associated with blood vessels, their free nerve endings can be found in periosteum, synovium and ligaments (172). Following injury, sympathetic fibers can modulate sensory nerve fiber function in several ways. There is evidence that in chronic pain conditions, sprouting of TrkA$^+$ sensory fibers is accompanied by increased growth of nearby sympathetic fibers (180). Additionally, noradrenaline released from the newly sprouted sympathetic fibers alters the nociceptors ion channel properties to make them more readily activated by painful stimuli (181).

2.2 RHEUMATOID ARTHRITIS

2.2.1 Epidemiology

Rheumatoid arthritis is a systemic, autoimmune disease with an unclear etiology. Genetic, environmental, and immunological factors all contribute to the development of RA (182). In industrialized countries RA affects 0.5% to 1% of the population, with an approximately three-

fold higher incidence among middle-aged women than men (183,184). Positive family history increases the risk of RA three to five times and twin studies have found above 50% contribution of genetic factors for the entire disease (185). Genome wide association studies (GWAS) and large-scale cohorts have identified more than a hundred high risk loci for RA, most of which are strongly related to immune mechanisms (186–188). Examples of genetic risk factors are HLA DRB1 alleles and a single nucleotide polymorphism (SNP) in PTPN22 gene (189,190). Interestingly, data indicate that these risk alleles are associated with a subset of RA characterized by the presence of ACPAs or rheumatoid factor (RF, anti-IgG antibody), or both. Development of RA is strongly associated with environmental factors. Cigarette smoking is the greatest environmental risk factor for RA (191,192), which doubles the chance of RA development. Additionally, RA is associated with periodontal disease (193), gastrointestinal dysbiosis (194,195) and obesity (196), but the exact relationships between these factors and disease development remain to be elucidated.

2.2.2 Pathogenesis of rheumatoid arthritis

RA is a heterogeneous disease, which encompasses various disease subsets that can differ in their underlying pathophysiology (182). Through common inflammatory pathways these subsets lead to a similar phenotype in most of the patients, that is characterized by joint edema, synovitis (synovial inflammation and hyperplasia), cartilage damage, bone erosion and pain. Small and medium sized joints in hands and feet are usually affected first. If left untreated, the disease can spread to large joints like hips or knees and lead to severe physical disability and significantly reduced quality of life.

Several cell types of both the innate and adaptive system are involved in the pathophysiology of RA. The innate immune response includes macrophages, mast cells, and dendritic cells, which produce a cascade of inflammatory mediators. These factors can then further attract a variety of mononuclear cells, to the site of the injury, where they overproduce pro-inflammatory cytokines, prostaglandins and oxygen-reactive species (197). Several cytokines play a fundamental role in the pathophysiology of RA, with TNF-α, IL-1β, IL-6 and IL-17 being the key molecules driving inflammation and bone destruction (198).

2.2.3 Pain in rheumatoid arthritis

Patients suffering from rheumatoid arthritis identify joint pain as one of their most problematic symptoms and the major reason for seeking medical help (199). RA pain has a wide range of characteristics (constant, intermittent, localized or widespread) and varies in intensity along the

course of the disease. Classically, RA pain has been associated with inflammation and tissue injury. However, increasing evidence indicates that RA-associated pain arises from both inflammatory and non-inflammatory mechanisms. Non-inflammatory mechanisms of RA-associated pain include structural and neurochemical changes within the joint and sensory nervous system or altered pain processing in the CNS (200,201). The contribution of (seemingly) inflammatory-independent mechanisms in RA pain is depicted by the observation that 19-37% of RA patients present with neuropathic-like (nerve-injury) pain features (202,203). In addition, the prevalence of fibromyalgia (widespread pain) was shown to be higher in RA patients (14-24%) compared to the general population (2-4%) (204). This strong co-morbidity as well as the higher incidence of widespread pain in patients with long-term RA suggests that disturbed pain modulation plays an important role in RA pain pathogenesis (205).

Further supporting the notion that the inflammatory process in the joint is not the sole driver of joint pain stems from the observation that a substantial proportion of RA patients (60%) do not experience satisfactory pain reduction and/or long-term pain suppression after successful anti-inflammatory treatment with disease-modifying anti-rheumatic drugs (DMARDs) (206–209). This lack of pain control raises the possibility that RA pain is only partly dependent on the inflammatory status. In fact, there is poor correlation between disease activity and pain (208,209). It is worth mentioning that during the development of RA, joint pain is frequently one of the first symptoms of an emerging disease, frequently present prior to visible join edema, suggesting that pain can develop prior to the RA diagnosis due to sub-clinical inflammation or via other mechanisms. In the context of this thesis, one important observation is that ACPAs, and other autoantibodies, can be present prior to disease onset (see paragraph 2.2.5). Furthermore, combination of arthralgia (joint pain) and the presence of ACPAs can be predictive of development of RA (210,211). We have previously identified a direct link between ACPA seropositivity and arthralgia (138). Wigerblad *et al*. found that injecting polyclonal ACPA causes mice to display long-lasting pain-like behaviors, without any signs of joint inflammation. In **Studies I-III** we have demonstrated that also other RA-associated monoclonal antibodies, with distinct mechanisms of action, induce mechanical hypersensitivity when injected to mice. These observations suggest that pain in RA could be driven through several mechanisms in the absence of joint inflammation.

2.2.4 Pain management in rheumatoid arthritis

Current pain management strategies for RA include directly acting analgesics or drugs that suppress joint damage and inflammation, and indirectly improve pain scores. Although modern

pharmaceuticals allow excellent disease control, pain continues to be a problem for a significant number of patients even one year post-treatment initiation (212).

Several types of analgesics are currently used for pain management in RA. These include non-steroid anti-inflammatory drugs (NSAIDs), paracetamol, opioids and neuromodulators (antidepressants and anticonvulsants). Although effective at relieving pain short-term, analgesics were shown to have limited efficacy, especially beyond 6 weeks (212). Additionally, prolonged treatment with analgesics is associated with high risk of adverse effects including gastrointestinal, renal, or cardiovascular, which increase with combined pharmacotherapy (213,214).

Disease-modifying anti-rheumatic drugs are a heterogeneous group of drugs categorized as synthetics (small molecules given orally) and biologics (proteins administered parenterally). They aim to decrease inflammation, swelling, limit the tissue damage and reduce joint pain within several weeks (215). Methotrexate (MTX) is the most widely used synthetic DMARD and is a first-line treatment option. When used with other DMARDs or glucocorticoids, MTX can provide almost half of the patients with proper disease control or even remission at early stage RA (216,217). Biologics are used to treat patients suffering from moderate and severe arthritis that did not respond to conventional DMARDs (218). The most frequently used biologics include decoy tumour necrosis factor (TNF) receptors (etanercept), anti-TNF antibodies (certolizumab), interleukin-1 antagonists (anakinra) and anti-interleukin 6 antibodies (siltuximab). Large cohort studies report that despite successful anti-inflammatory treatment with DMARDS, more than 70% of patients with established RA continue to suffer from pain that ranges from moderate to severe (206). A Swedish cohort study of early RA patients (EIRA) brought similar results (209). Almost one-third of the patients who responded well to MTX had remaining pain after three months of treatment. Importantly, the remaining pain was associated with high disability and low inflammation at baseline. These results further support the observations that pain in RA can be partially driven by non-inflammatory mechanisms.

2.2.5 Preclinical rheumatoid arthritis

Prior to the development of clinically diagnosed RA, patients often present with several RA-related abnormalities, which are termed preclinical RA or pre-RA. Pre-RA is often characterized by arthralgia, bone erosion and autoantibody production, all in the absence of detectable inflammation. Pre-RA patients can develop autoantibodies against several post-translational modifications (AMPAs) like ACPAs, anti-carbamylated (ACarPAs), anti-

acetylated (AAPAs) and malondialdehyde acetaldehyde (MAA) modified protein antibodies, rheumatoid factor (RF) and anti-collagen type II antibodies (anti-CII), which can be found in the serum many years before the onset of the disease, with titres rising closer to the time of diagnosis (219–226).

Although joints are the primary target of RA, the starting point of systemic autoimmunity is still unknown. A compelling amount of evidence points towards the lungs as a site where autoimmunity develops, however it still remains a question why inflammation is exclusively directed towards the joints. Importantly, seropositivity is not enough to induce chronic synovial inflammation, since not all ACPA+ individuals develop RA (227) and several animal studies show that passive ACPA transfer does not induce arthritis (138,228,229). A "second hit", such as trauma or infection may be necessary for autoimmune reaction to reach the joint (230–232). The preclinical phase of RA should be strongly considered a "window of opportunity" for the treatment of the disease and for that an increased understanding of the autoimmunity and its shift towards active RA is needed (233).
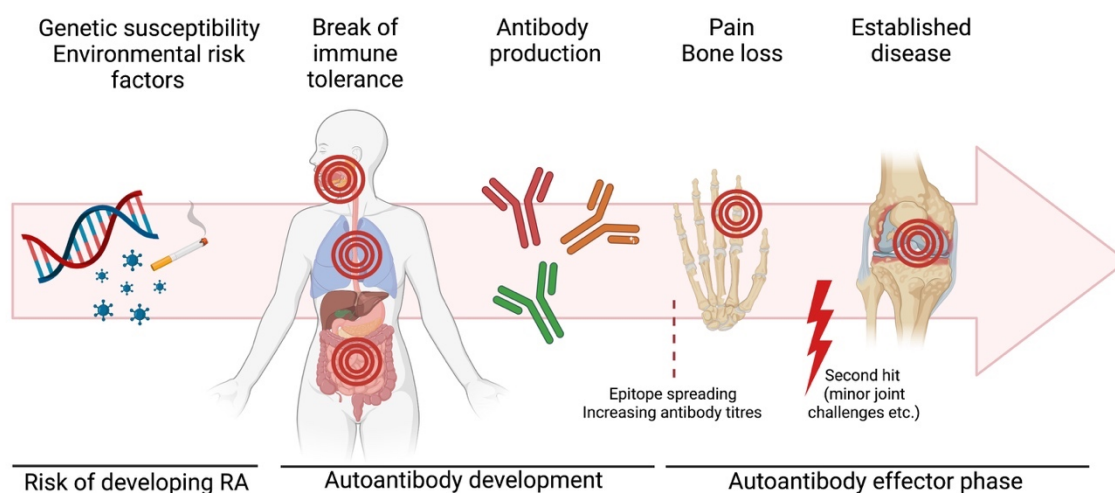

Figure 3. Schematic representing stages in the development of autoantibody-positive rheumatoid arthritis. When exposed to environmental factors like pollutants or microorganisms at the mucosal sites (oral cavity, lungs, intestine) genetically susceptible individual can develop local inflammation, which activates the immune system and might lead to the break of immune tolerance and generation of autoantibodies. Gradually, autoantibodies increase in titres and undergo epitope spreading which eventually might lead to bone erosion and pain, preceding any signs of joint inflammation. Minor joint challenges, called "second hits", might lead to chronic inflammation, which if left untreated can develop into established disease and cause significant joint destruction. Created with Biorender.com.

2.2.6 Autoantibodies in rheumatoid arthritis

Antibodies (Abs) are tetramer proteins (composed of four polypeptide chains) produced by B cells and plasma cells that bind and neutralize pathogens and elicit an immune response by recruiting other cells and molecules. They exist in two forms: as membrane-bound antigen

receptors on B cells or secreted proteins (234). Antibodies are composed of two light chains (L) and two heavy chains (H) with each chain containing a variable (Fab) and a constant (Fc) region. The variable domain of the antibody allows for unique antigen recognition and binding. The constant region binds to other molecules involved in pathogen elimination like Fc receptors and complement system proteins. Depending on the type of heavy chains, antibodies belong to different isotypes named IgA, IgD, IgE, IgG and IgM. Mouse IgG antibodies can be further subdivided into four subclasses, named in order of decreasing abundance: IgG1, IgG2a, IgG2b and IgG3. Each IgG subclass has unique properties including antibody binding, immune complex formation, complement activation and half-life (234). Antibodies directly bind their antigens, resulting in functional modifications of the antigen. However, they can also form immune complexes (ICs, a complex of antibodies bound to an antigen) that activate the complement system and Fc receptors (FcRs).
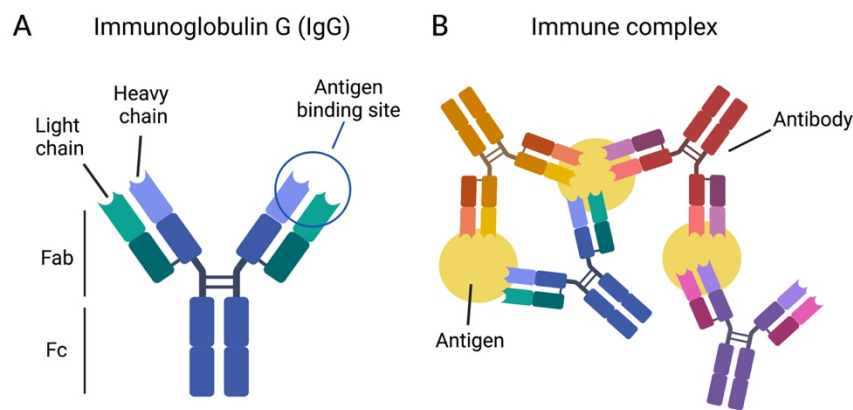

Figure 4. Immunoglobulin structure. **(A)** IgGs are glycoproteins composed of two identical heavy chains (blue) and light chains (green). The variable regions (lighter blue and green) form the Fab fragment which recognizes and binds antigens. The constant regions of the heavy chains form the Fc part, which mediates the effector functions of IgG. **(B)** Immune complex (IC), also called antigen-antibody complex is a molecule formed from binding multiple antigens to antibodies, needed to activate FcγRs. Created with Biorender.com.

The immune system enables antibodies to distinguish between foreign and self-molecules, a phenomenon called immunological tolerance (234). During the process of maturation, autoreactive B cells are selected and eliminated from the system in order to prevent the production of antibodies that recognize self-antigens (autoantibodies). Deficient regulation of the tolerance might lead to autoimmune diseases where autoantibodies are produced and can cause tissue damage.

High titres of autoantibodies are present in several autoimmune diseases and can serve as serological markers, however often their pathophysiological relevance is unknown. Rheumatoid arthritis is characterized by the presence of several autoantibodies including ACPAs, ACarPAs and AAPAs, rheumatoid factor and anti-collagen II antibodies (anti-CII). Although ACPAs, ACarPAs and AAPAs are considered three independent classes of

autoantibodies (235), they often occur in parallel in RA and cross-react on both poly- and monoclonal level (236–239), therefore they may be considered as one group of anti-modified protein antibodies (AMPAs) (240). Knowledge about the direct pathogenic potential of antibodies in RA comes from observations that arthritis is mediated in experimental animals via B cells (241) as well as by passive transfer of autoantibodies (242).

Rheumatoid factor (mainly IgM, but also to some extent IgG and IgA) is an antibody recognizing the Fc portion of IgG, which is present in up to 80% of RA patients (243). RF forms large immune complexes with Fc part of IgG that are potentially involved in RA pathogenesis. For a long time, RF was considered the only serological marker of RA and several studies have shown its association with a more severe disease phenotype (244,245). Furthermore, high titres of RF are correlated with the risk of developing RA (246). However, recent studies have shown that RF is not specific for RA and it can be found in patients with other autoimmune or infectious diseases as well as in 15% of healthy individuals (247).

Citrullination, also known as deamination, is a post-translational protein modification (PTM) that occurs when peptidyl-arginine deiminase enzymes (PADs) convert arginine into citrulline, in the presence of high calcium levels. Citrullination results in significant conformational and biochemical changes of the proteins and renders them immunogenic leading to the breach of self-tolerance (248,249). Anti-citrullinated protein antibodies have been first reported in the serum of RA patients more than twenty years ago (250). To date, several ACPA autoantigens have been identified including citrullinated fibrinogen, vimentin, histones, alpha-enolase and collagen type II (251–254), however due to significant cross-reactivity, ACPAs most likely also bind to other, yet unidentified citrullinated self-antigens (226,237,239,255). ACPAs are found in approximately 60-70% of RA patients and as described in paragraph 2.2.5, can be detected in serum many years before the disease onset (256,257). Given their specificity for RA, ACPAs are a valuable diagnostic tool for diagnosing established RA as well as identifying individuals at risk (258,259). Furthermore, an increasing amount of evidence shows that ACPAs play a significant role in mediating the pathogenesis and development of RA (260,261).

Carbamylation (also referred to as homocitrullination) is, unlike citrullination which is catalyzed by an enzyme, a chemical modification in which lysines are converted into homocitrullines under the influence of cyanate. Carbamylation is an irreversible modification and affects mostly long-lived proteins due to their ability to acquire homocitrulline residues over time (223). The carbamylation of amine groups changes the charge of the molecules which in turn allows the carbamylated derivatives to acquire antigenic properties. Carbamylated

proteins are thought to play a role in inflammation as they can modulate the function of inflammatory cells *e.g.* enhance the production of metalloproteinase-9 (MMP-9) by monocytes (262). ACarPAs can be found in the serum of 45% of RA patients (223). Although they partially overlap with the presence of ACPAs they can occur also in ACPA negative RA patients (223). The exact autoantigens recognized by ACarPAs are still being investigated. Among potential proteins, fibrinogen, which is a frequent target of ACPAs, was shown to be highly accessible for carbamylation (263) and the presence of antibodies against carbamylated fibrinogen was confirmed in RA patients (263). ACArPAs were shown to be associated with progression to RA as ACArPAs positive individuals with arthalgia developed RA within a shorter time than individuals with RF of ACPAs (264). This association was maintained even in the double positive ACPA/ACArPA arthalgia individuals suggesting that ACArPAs could serve as an additional biomarker in predicting the development of RA (264).

The fibrillar collagen type II (CII) is the major protein building articular cartilage. Depending on the type of assay and target epitopes used, anti-CII antibodies are found in the blood of 3-27% of RA patients, with their levels peaking at the time of diagnosis (265,266). Anti-CII autoantibodies are strongly associated with active inflammation during the early stages of RA and their levels decrease within the first few years of the disease. RA patients positive for anti-CII antibodies have a distinct phenotype, characterized by higher disease activity and an early acute phase response (267,268). The role of anti-CII antibodies in initiating arthritis has been reported in several species, including mice (269,270). Additionally, thanks to targeting a joint specific epitope, anti-CII antibodies can serve as a tool to study the effector phase of the disease (271).

2.2.7 Fc-gamma receptors

Fc gamma receptors (FcγR) are membrane-bound glycoproteins, which bind the Fc region of IgG antibodies and are predominantly expressed in immune cells. FcγRs regulate innate and adaptive immune responses involving phagocytosis, cytolysis, cytokine production, immune complex clearance and antibody transport (272,273). In rodents FcγRI, FcγRIII and FcγRIV are activating receptors and FcγRIIb is inhibitory (273). Activating receptors are characterized by a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) signaling domain, whereas FcγRIIb contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling domain. FcγRs bind the four subclasses of mouse IgG, IgG1, IgG2a, IgG2b and IgG3, with different affinities (274,275). FcγRI is a high-affinity receptor binding murine IgG2a in both

monomeric and IC form, whereas other FcγRs are activated only by aggregated IgG ICs and show a broader IgG subclass specificity (276).

Recent studies show that FcγRs can be expressed not only on immune cells but also on neurons (277,278) and osteoclasts (279,280) and their functional significance in these cell types is under investigation. For example, FcγRs expressed on osteoclasts (bone resorbing cells) were shown to contribute to bone homeostasis by regulating osteoclastogenesis (new osteoclast formation) (281). Under autoimmune inflammatory conditions characterized by abundance of IgG ICs, like rheumatoid arthritis, a disruption in the balance between activating and inhibiting FcγRs was shown to drive bone erosion (280). Furthermore, in a murine serum transfer arthritis model, deletion of FcγRIV was shown to protect the mice from bone erosion induced by inflammation (282). On the other hand, the exact functional role of FcγRs on neurons is unknown. The presence of FcγRI on rat sensory neurons was shown to be crucial for both *in vitro* and *in vivo* neuronal activation (measured as increased calcium, substance P and CGRP release) after stimulation with ICs (277,278,283–285). Furthermore, findings from our group indicate the presence of both FcγRI and FcγRIIb on naïve murine sensory neurons and that neuronal FcγRI mediate nociception after injection of anti-CII antibodies prior to development of joint edema in mice (285). Changes in neuronal FcγR expression might play an important regulatory function in response to injury or inflammation. Increased capacity of binding immune complexes could lead to enhanced neuronal excitability and result in hypersensitivity.

2.2.8 Bone erosion in rheumatoid arthritis

Destruction of bone is a pathological hallmark of RA that is reflective of disease severity and associated with poor outcomes. RA-induced bone erosion is characterized primarily by local, periarticular erosions, but also systemic bone loss (286). Bone loss is caused by excessive resorptive osteoclast activity and inadequate bone formation by bone-forming osteoblasts (287). Although bone resorption was believed to reflect the inflammatory burden in the joint, many RA patients have low bone mass at diagnosis, suggesting that erosion precedes inflammation (288,289). Clinical observations have found evidence that disrupted bone metabolism and thinning of cortical bone is already present at the pre-RA stage (290–292). Additionally, the subset of RA patients positive for ACPA is characterized by a higher degree of joint destruction and a much more severe course of the disease, compared to ACPA negative patients (293–295). Furthermore, despite successful treatment with DMARDs, progression of joint damage is still present in one-quarter of RA patients, which can be explained by the fact that not all DMARDs exhibit significant effects on ACPA levels in the blood (296,297).

Preclinical murine research has demonstrated that polyclonal ACPA IgG and certain monoclonal ACPA IgG binds osteoclasts *in vitro* (229,298) and osteoclast precursors *in vivo* (138). Binding of normally expressed citrullinated epitopes on osteoclasts is thought to promote osteoclastogenesis, leading to increased bone resorption (228,229). Besides directly binding citrullinated epitopes or FcγRs on osteoclasts surface, ACPA-containing immune complexes can stimulate other cells to release pro-inflammatory cytokines that increase osteoclastogenesis and lead to subsequent bone loss (299–301). However, it has been shown that ACPA can induce bone erosion also in the absence of joint inflammation (138,229). When stimulated with polyclonal ACPAs, both human and murine osteoclasts secrete CXCL1, which then further activates them through an autocrine loop (138,229). Blocking CXCL1, or its receptor, completely inhibits osteoclastogenesis and resorption, preventing bone loss *in vitro* and *in vivo* (138,229). Interestingly, CXCL1 blockade inhibits also pain-like behavior seen in mice after polyclonal (138), and monoclonal ACPA injection (**Study II**), suggesting a link between increased osteoclast activity and pain. Importantly, in **Study I** we have shown that also other RA-associated autoantibodies have the capacity to induce pain-like behavior and bone erosion in the absence of overt joint inflammation. A combination of monoclonal AMPA IgG and a monoclonal IgG without detectable citrulline specificity induces bone erosion *in vivo* and osteoclastogenesis *in vitro* (155). Furthermore, bone erosion induced by these two monoclonal IgGs is accompanied by increased levels of lysophosphatidylcholine 16:0 (LPC 16:0) in bone marrow and increased expression of two secretory phospholipase A2 isoforms (*Pla2g2* and *Pla2g5*) in the ankle joint. Inhibiting sPLA$_2$ reverses both mechanical hypersensitivity and bone erosion induced by these mAbs. These findings suggest that RA-associated autoantibodies stimulate osteoclasts to release various factors (*e.g.* CXCL1, lipids), which in turn can sensitize the nociceptors and lead to mechanical hypersensitivity in the absence of joint inflammation.

2.3 FIBROMYALGIA

2.3.1 Epidemiology of fibromyalgia

Fibromyalgia syndrome (FMS), classified as nociplastic pain, is a common and complex disorder characterized by chronic, widespread, muscular and joint pain as well as generalized hypersensitivity to sensory stimuli (302). It is often accompanied by fatigue, unrefreshing sleep, anxiety, depression and cognitive difficulties. Depending on the diagnostic criteria, the prevalence of FMS ranges between 2-8% in general population with a higher incidence among females than males (303,304). Fibromyalgia has a high negative impact on the quality of life as it leads to personal suffering, loss of productivity, substantial disability and high use of medical resources (305). Disease can develop at any age, including childhood (306), however the prevalence rises linearly with increasing age and is the highest in elderly women (303). FMS may be triggered by physical trauma, surgery, significant psychological stress or certain infections like Lyme disease or Epstein-Barr virus (307). In other cases, symptoms develop spontaneously and accumulate over time, without a certain trigger. Importantly, patients with FMS often have a history of localized chronic pain *e.g.* headaches, IBS, endometriosis or back pain (308) and the long duration of regional pain (> 6 years) was identified as a risk factor for FMS development (309). Of note, comorbid FMS occurs in approximately 10-30% of patients with other rheumatic disorders (*e.g.* RA, OA, systemic lupus erythematosus (SLE), psoriatic arthritis) (310,311).

Various studies have shown potential involvement of genetic factors in FMS development (312). Indeed, first-degree relatives were shown to have a higher risk of developing FMS and reduced pressure pain thresholds, compared to general population (313–315). GWAS studies have identified several genetic polymorphisms associated with FMS development including ones in TRPV2 gene (315), serotonin transporter (5-HTT) (316), catechol-O-methyltransferase (COMT) (317) and serotonin 2A (5-HT2A) genes (318). It has been proposed that these polymorphisms might contribute to increased pain severity and psychiatric symptoms seen in FMS patients, however further studies are needed to understand their exact role in susceptibility to FMS (312).

2.3.2 Pathogenesis of fibromyalgia

Fibromyalgia encompasses a wide range of symptoms and functional alterations in many systems. Although the exact pathogenesis is still unknown and further complicated by many

co-morbidities, it is thought to involve an interaction between central and peripheral mechanisms.

2.3.2.1 Central pathologies in fibromyalgia

Several studies have implicated that abnormal central pain processing is the key mechanism of FMS pathophysiology. Indeed, patients with FMS were reported to exhibit allodynia/hyperalgesia in response to multiple somatosensory modalities including innocuous cold, warmth or pressure pain (319). Additionally, their responses to painful stimuli are characterized by increased temporal summation of second pain (also known as "wind-up") (320) and pain referral to larger areas (321), both reflecting increased excitability of spinal cord neurons, a cardinal feature of central sensitization. Moreover, several behavioral studies have shown that FMS patients have impaired descending pain inhibition associated with decreased activation of rostral anterior cingulate cortex (rACC) and brainstem - brain regions which are part of central pain regulatory system (322–324). In addition, brain imaging studies indicate that brain areas involved in pain inhibition display a less functional connectivity in FMS patients, compared to healthy controls (325,326). Apart from functional disturbances, FMS is shown to be associated with atrophy of gray matter in brain regions responsible for pain processing. Although the morphological changes do not seem to correlate with pain symptoms, they might be a precondition for central sensitization (327,328). FMS was also shown to be associated with several neurochemical changes in the CNS, including increased levels of glutamate and decreased levels of GABA in the insula and decreased number of μ-opioid receptor (MOR) in pain-processing structures like ACC (329–331). Moreover, factors involved in pain transmission and central sensitization such as substance P (332), NGF (333) and BDNF (334) were found to be elevated in patients' cerebrospinal fluid (CSF).

In addition to neuronal alterations in FMS, overactivation of glia cells in the CNS was shown to be involved in the pathophysiology of the disease due to its facilitatory role in central sensitization. A recent study using positron emission tomography (PET) demonstrated increased glia activation in the brain cortex of FMS patients, compared to healthy controls (335). Researchers have measured the binding of translocator protein (TSPO), a protein widely upregulated in the activated microglia and astrocytes (336) and reported that higher TSPO signal in the brain correlates with fatigue in the studied FMS cohort. By measuring the levels of monoaminoxidase-B (MAO-B), protein specifically expressed in the astrocytes, researchers suggested the predominant role of microglia in FMS pathology. Activated glia is thought to be the source of elevated concentrations of pro-inflammatory cytokines in the CSF of FMS

patients. Several studies demonstrated increased CSF levels of factors involved in neuroglia signaling like IL-8 or fractalkine (337–339). Furthermore, rodent equivalent of IL-8 (CXCL1) co-localizes with TSPO in glia cells and TSPO agonists modulate the expression of CXCL1 (340), supporting the hypothesis of TSPO-glia associated mechanisms in FMS.

2.3.2.2 *Peripheral pathologies in fibromyalgia*

Although FMS was traditionally thought to be a central pain disorder, increasing data show that FMS patients also present with peripheral pathologies. Importantly, individuals with FMS were reported to have a reduced number of intraepidermal nerve fibers in the skin, specifically C-afferent fibers (341–343). This was shown to be associated with a more severe disease phenotype and higher symptom load (344). Furthermore, using microneurography, it has been demonstrated that mechano-insensitive C nociceptors (classified as silent nociceptors) in FMS patients have an abnormally high slowing of conduction velocity and many of them respond to mechanical stimulation and exhibit spontaneous activity (345). Researchers have suggested that these pathological features seen in C-fibers are likely due to decreased axon diameter. Indeed, an ultrastructural study of the skin innervation in FMS patients reported morphological changes (termed "ballooning") in the unmyelinating Schwann cells, which was associated with a more peripheral localization of the axons within the Schwann cell sheath as well as smaller axon diameter (346). Intriguingly, these abnormalities were, so far, not reported in any other pain condition.

Individuals with FMS suffer from increased muscle fatigue and a low tolerance to exercise (347). This clinical observation led to a hypothesis that FMS is associated with muscle pathology. Although no signs of degeneration, regeneration or inflammation can be found in FMS muscles, studies have shown that FMS is associated with lower capillary density in the muscles (348), mitochondrial dysfunction (349) and lower oxygenation of the muscles (350). Furthermore, microdialysis studies have shown increased interstitial levels of glutamate, pyruvate and lactate levels in the muscles of FMS patients (351). Intriguingly, a 15-week exercise intervention normalized these alterations and decreased pain intensity. Moreover, decreased levels of ATP in the muscles (352), but also in skin (353) and peripheral blood mononuclear cells (354) of FMS individuals were reported, pointing to global metabolic alterations in FMS.

2.3.2.3 Inflammation and immunity in fibromyalgia

An altered balance between pro- and anti-inflammatory cytokines is thought to play a role in induction and maintenance of pain. Several studies have examined the systemic levels of cytokines in patients with FMS, however results have been so far conflicting due to different methods used to detect these factors. Systematic reviews performed by Ücelyer et al. and Andrés-Rodriguez et al. have shown that FMS patients have higher serum and plasma levels of IL-6, IL-8, IL-1Ra and IL-17A, compared to controls, but the levels of IL-1β, TNF, IFNγ or IL-10 are not altered (355,356). Interestingly, the levels of anti-inflammatory cytokine IL-4 were shown to be increased in FMS patients, probably as a counterbalance to increased levels of IL-6 and IL-17A (357). More studies are needed to understand if these cytokines mediate symptoms in FMS or rather could be used as symptom biomarkers in FMS.

The high comorbidity of FMS in other autoimmune disorders like SLE, RA or thyroid autoimmunity as well as the precipitation of symptoms in response to infection highlights the role of autoimmunity in the pathophysiology of the disease. More than twenty years ago several independent studies have reported occurrence of various autoantibodies in the serum of FMS patients. The most frequent autoantibody group found in FMS patients were the anti-nuclear antibodies (ANA), which were consistently reported in several patient studies (358–362). Anti-serotonin, anti-ganglioside and antiphospholipid antibodies were also shown to be higher in patients with FMS as well as in patients with chronic fatigue syndrome (363,364). Intriguingly, family members of these patients also had these antibodies strengthening the hypothesis of genetic predisposition to the disease (364). Although researchers suggested an association between autoantibodies and different FMS symptoms including cognitive dysfunction and hypersomnia (362), the clinical relevance of the autoantibodies has not been established so far. In **Study IV** we have provided evidence that IgG isolated from FMS patients recapitulates key sensory, motor and anatomical features of FMS (mechanical and thermal hypersensitivity, tenderness, decreased skin innervation and reduced activity) when injected into mice (365). Importantly, we have demonstrated that FMS IgG binds SGCs and neurons in mouse and human DRGs, which suggests that FMS patients indeed have autoreactive antibodies. Future studies are needed to unravel the exact reactivities of the autoantibodies as well as the underlying cellular and molecular mechanisms behind FMS IgG-induced sensitization.

2.3.3 Pain management in fibromyalgia

Fibromyalgia is a multidimensional disease and although patients share similar symptoms, they require individualized treatment based on the associated conditions and the patients' previous

experiences (366). Although currently there is no cure for FMS, there is some evidence for pain-reducing effects of centrally acting medications like antidepressants (duloxetine, milnacipram) and anticonvulsants (pregabalin), but many FMS patients do not benefit from these drugs (367). Opioids were shown to have very limited efficacy in FMS treatment (368). Although patients have altered endogenous opioid signaling with decreased receptor availability, high levels of opioids are found in their biological fluids, which is probably why opioids are not effective. A combination of tramadol, a weak μ-opioid receptor agonist with mild serotonin-norepinephrine reuptake inhibitor (SNRI) activity, and paracetamol was shown to improve the global quality of life, however tramadol alone had no benefit on treatment of FMS symptoms, compared to placebo (369). The effects of pain treatment with classical analgesic drugs like paracetamol and NSAIDs were so far disappointing (370), however they can be used to treat concurrent disorders *e.g.* OA or RA (371). Despite a better understanding of FMS pathogenesis and increasing number of pharmaceuticals available for FMS treatment, pharmacological treatment alone often demonstrates insufficient symptom relief and thus should be a part of multidisciplinary therapeutic approach (372). Exercise is recognized as an important part of FMS management as both aerobic exercise as well as resistance training were associated with significant improvement in pain and physical function (367). Additionally, in association with exercise, cognitive behavioral therapy is strongly recommended especially in patients who experience depression and anxiety along with FMS (373).

Our recent findings (**Study IV**) demonstrating that FMS might be, at least partially, mediated by autoreactive antibodies provides an intriguing therapeutic opportunity. Confirming the role of autoantibodies in FMS pathogenesis might allow for using therapies which reduce the total titers of autoreactive IgG (plasmapheresis) or interfere with binding of the autoreactive antibodies. Of note, a study done in 2008 has demonstrated that a short treatment with intravenous immunoglobulin (IVIG) significantly improved pain, tenderness and strength scores in FMS patients without inducing significant adverse effects (374), proving that immunomodulating therapies could be of benefit in FMS.

2.4 SUMMARY

In conclusion, increasing evidence points to novel roles of autoantibodies in pronociceptive processes in diverse conditions including rheumatoid arthritis and fibromyalgia. Although symptoms like inflammation and joint destruction are satisfactorily managed in the clinic, pain continues to be a problem for a significant percent of patients, pointing to inflammation-independent mechanisms that drive hypersensitivity. Recent studies have demonstrated that

autoantibodies can exert their pronociceptive functions through various mechanisms *e.g.* by signaling at Fc receptors, binding self-reactive targets or recruiting complement. This thesis provides further knowledge on the complex mechanisms through which RA- and FMS-associated autoantibodies contribute to pain. Delineating the interactions between the immune and sensory system will hopefully open new perspectives and lead to the identification of new targets for development of novel analgesics and therapeutic strategies in painful autoimmune conditions.
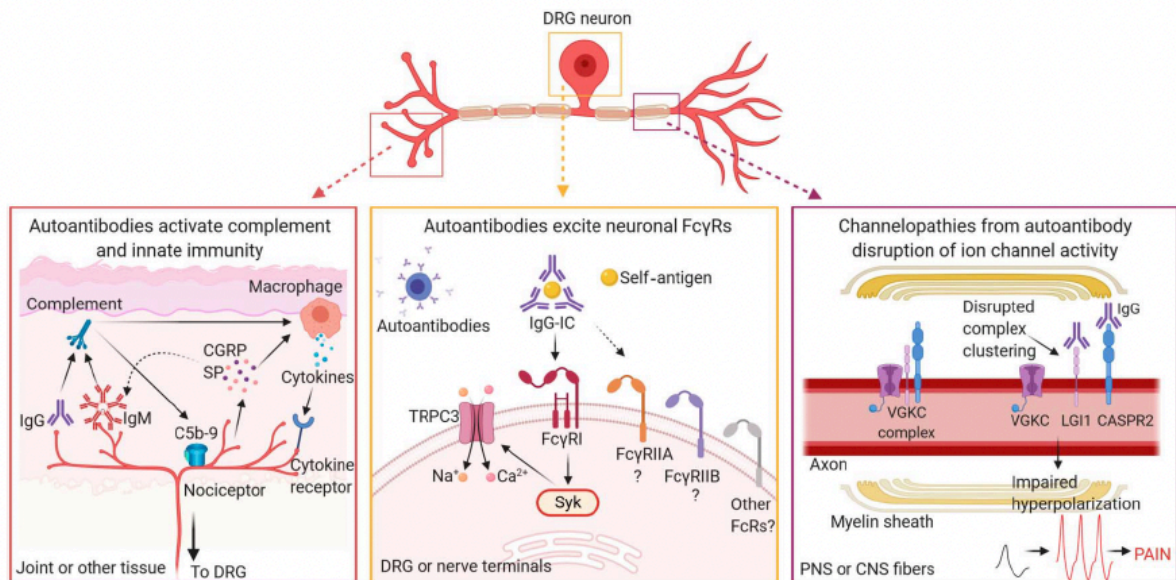

Figure 5. Autoantibodies can cause hypersensitivity and pain through multiple mechanisms. Presence of IgG or IgM autoantibodies in tissues like joint can stimulate the complement system and lead to inflammation. At the level of DRG or its nerve terminal, IgGs in immune complex can activate neuronally-expressed FcγRs resulting in neuronal activation. Autoantibodies can also disrupt the activity of voltage gated ion channels (VGKC) located on the axons leading to disturbed hyperpolarizing currents which in consequence increase action potential firing and cause pain. Reprinted with permission and adapted from Lacagnina et al. 2021.

3 RESEARCH AIMS

This thesis has the overall aim to explore the mechanisms through which autoantibodies drive persistent pain in conditions like rheumatoid arthritis and fibromyalgia, and to challenge the classical view that autoantibodies induce pain solely by initiating inflammatory processes.

Thus, the specific aims of this thesis are:

Study I: To examine the contribution of osteoclast activity to mechanical hypersensitivity induced by a combination of two RA patient-derived monoclonal autoantibodies

Study II: To explore the mechanisms through which a single monoclonal osteoclast-activating anti-citrullinated protein antibody (ACPA) induces pain-like behavior in mice

Study III: To investigate the pronociceptive properties of a monoclonal AMPA, which lacks osteoclast-activating characteristics

Study IV: To understand if autoreactive IgGs are responsible for key symptoms of fibromyalgia and to scrutinize the mechanisms behind their pronociceptive properties in mice

4 MATERIALS AND METHODS

4.1 PATIENTS

Synovial fluid and serum collection for **Study I – IV** were done under the approval from the regional ethics committees (Sweden and UK). Samples from patients were collected following written informed consent. Patients with RA fulfilled RA criteria as defined by the American College of Rheumatology 1987. Fibromyalgia syndrome (FMS) patients fulfilled both 1990 and 2011 ACR diagnostic criteria for FMS and were unaffected by other sensory, autoimmune or rheumatological conditions. Human DRG was collected from a 53-year-old female donor (without a history of chronic pain, who died from head trauma), following consent from the family and under the approval of McGill University Health Centre Review Board.

4.2 ANIMAL MODELS

4.2.1 Animals

All animal procedures were approved by the local ethical committees for animal research in Sweden (Stockholm Norra Djurförsöksetiska nämnd), France (Comité Régional d'éthique en matière d'expérimentation animale Auvergne) and UK (King's College London Animal Welfare and Ethical Review Body under a UK Home Office Project License). All work was done in agreement with the Animal Research Reporting of *In Vivo* Experiments (ARRIVE), IASP Guidelines as well as the UK Home Office Animal Procedures 1986 Act. Mice were housed in standard cages (3-5 per cage) in a temperature-controlled room with a 12-hour light/dark cycle, with food and water *ad libitum*.

Different mouse strains were used for the work in this thesis. For **Study I** female BALB/c (10-12 weeks old) mice were purchased from Janvier labs (Le Genest-Saint-Isle, France) or Charles River (Freiberg, Germany). C57BL/6J WT-littermates and ASIC3 knock-out female mice were bred in University Clermont Auvergne Medical School animal facility (France) and housed as described above. For APETx2 pharmacological experiment, C57BL/6J female mice were purchased from Janvier labs (Le Genest-Saint-Isle, France). For **Study II and III** male and female BALB/c (10-12 weeks) from Janvier labs were used. Additionally in **Study III** male and female BALB/c WT and FcRγ $^{-/-}$ mice (lacking the activating receptors FcγRI, III, and IV), which originated from the same breeding line, were maintained as homozygous mice in parallel and bred at the Comparative Medicine Biomedicum, Karolinska Institutet. C57Bl/6 male and female mice used for flow cytometry experiments were bred and maintained at the

animal facility of University Medical Center Utrecht. **Study IV** used C57BL/6J (8-10 weeks old) female mice purchased from Envigo UK Ltd and BALB/c (16 weeks old) female mice from Janvier labs.

4.2.2 Antibodies

4.2.2.1 Monoclonal IgG from RA patients

Monoclonal Abs (mAbs) used in **Studies I-III** were isolated from synovial fluid samples drawn from knee joints of RA patients who attended the Rheumatology clinic at Karolinska University Hospital and fulfilled RA criteria as defined by the American College of Rheumatology 1987. Monoclonal Abs were isolated from single memory B cells or antibody secreting cells and expressed as recombinant IgG1, as previously described (232,238,375). All IgGs underwent extensive quality control testing including specificity evaluation, size exclusion chromatography aggregation test and endotoxin test (<10EU/mg IgG). Murine chimera antibody of the human monoclonal 1325:04C03 was generated by replacing the human gamma and lambda/kappa constant regions with the murine IgG2a constant regions. 1325:01B02 mAb used in **Study I** is negative for reactivity towards all screened citrulline and arginine-containing peptides but does not show polyreactivity or general non-specific reactivity. Although it was previously shown to have functional activity and osteoclast binding (229) it is currently not known which epitopes 1325:01B02 mAb binds to. 1325:01B09 mAb used in **Study I and III** is classified as an anti-modified protein antibody (AMPA) as it binds to citrullinated, acetylated and carbamylated peptides. 1325:04C03 mAb used in **Study II** binds to citrullinated and carbamylated vimentin. Control mAbs used in **Studies I-III** (1276:01G09, 1362:01E02, and MGO53:0110) have no detectable reactivity.

Each mAb was injected intravenously alone at a dose of 2 mg/mouse or in combination (B02/B09) 1 mg/each mAb per mouse. Intra-articular injections were done with human 1325:01B09, 1325:04C03 and 1276:01G09 mAbs at a dose of 2.5 μg/joint in a 2.5 μl PBS volume.

4.2.2.2 Anti-collagen type II antibodies

For **Study I**, the collagen antibody-induced arthritis (CAIA) model was used. BALB/c female mice were injected i.v. with anti-collagen type II (CII) arthritogenic cocktail (1.5 mg/mouse, Chondrex) on day 0. Lipopolysaccharide (LPS, 25 μg/mouse, Chondrex) was injected intraperitoneally (i.p.) on day 5 to synchronize the onset of joint inflammation. Control group

received saline solution i.v. on day 0 and i.p. on day 5. Mice were sacrificed 16 days post CAIA-induction at the peak of inflammation.

4.2.2.3 *IgG from fibromyalgia patients*

Serum IgG was purified from patients with fibromyalgia (FMS) and healthy controls (HC). Sera for pooled sample testing were purified using HiTrap Protein G HP columns (GE Healthcare), eluted with 0.1 M glycine/HCl pH 2.7, and the pH adjusted to 7.4 with 1 M Tris pH 9; consequently, samples were dialyzed against PBS, concentration adjusted, stored at –20°C, and later thawed, pooled, and concentration-adapted using PBS-prewet concentration columns (Pall Corporation, Macrosep, 10K). Concentration measurements were performed using Nanodrop 2000 (ThermoFischer Scientific). The concentration of IgG-depleted sera (flow-through), which served as an additional control for FMS and HC IgG, was adapted before injection. Mice were injected i.p. with 8 mg of pooled FMS or HC IgG.

4.2.3 Drugs and drug delivery

4.2.3.1 *Analgesics*

For **Studies I-III** several different pain-relieving drugs with distinct mechanisms of action were used. Naproxen (50 mg/kg, Sigma), a non-selective, non-steroidal anti-inflammatory drug (NSAID) was administered as a single i.p. injection (**Study II**) or in repeated i.p./s.c. injections (**Study I and III** respectively) on three consecutive days post-antibody injection. Diclofenac (30 mg/kg, EliLily), a selective COX-2 inhibitor was administered as a single i.p. injection in **Study II and III**. Paracetamol (200 mg/kg), an analgesic and anti-pyretic was administered in **Study I** in a single i.p. injection. Two opioids, morphine (3 mg/kg, Sigma, s.c.) and buprenorphine (0.1 mg/kg, Sigma, i.p.) were given in a single bolus injection in **Study I** and **II, III** respectively. Gabapentin (50 mg/kg, Teva), an anticonvulsant with analgesic properties, used for treatment of neuropathic pain, was administered in a single i.p. injection in **Study II**.

4.2.3.2 *Osteoclast inhibitors*

Two osteoclast-inhibitors, zoledronate and tanshinone II A sulfonic sodium (T06) were tested for this thesis. Zoledronate is a nitrogen-containing bisphosphonate, which leads to osteoclast apoptosis and prevents bone mineral dissolution and matrix digestion. It is used in the clinic for treatment of osteoporosis, Paget's disease, osteogenesis imperfecta and bone malignancies (376). **For Study I and II** zoledronate (100 mg/kg, Sigma) was administered i.p. every 3 days and saline was used as vehicle control. T06 (kindly provided by Dr. Dieter Brömme) is a

derivative of natural component of Salvia miltiorrhiza, a herb commonly used in traditional Chinese medicine as an anti-osteoporotic compound (377). It selectively binds to ectosteric site of cathepsin K and prevents osteoclasts from degrading collagen (378). T06 was given by oral gavage in a 100 µl per 10 g body weight volume, every day for the whole duration of the experiment (**Study I**).

4.2.3.3 ASIC3 inhibitor

APETx2 is a peptide toxin isolated from a sea anemone *Anthopleura elegantissima* that selectively blocks ASIC3 homomeric and heteromeric channels, and has no effect on other ASIC channels (379). In **Study I**, APETx2 (Alomone Labs, 20 µmol in 10 µl saline) was injected intra-articularly (i.a.) into the ipsilateral ankle joint. The contralateral paw, injected with saline, was used as an internal control.

4.2.3.4 V-ATPase inhibitor

Bafilomycin A1 is a selective V-ATPase inhibitor, which prevents osteoclasts from secreting protons and subsequently attenuates acidification (380). In **Study I**, BafA1 (Abcam, 25 µg/kg) was administered s.c. daily for 5 days. Vehicle (0.01% DMSO in PBS) or 1362:01E02 mAb were used as controls.

4.2.3.5 CXCR1/2 antagonist

Reparixin L-lysine salt is a non-competitive allosteric inhibitor of CXCR1/2 that prevents receptor signaling by modifying its conformation (381). In **Study II**, mice were treated with s.c. injections of reparixin (MedChem Express), twice a day (30 mg/kg/day, in 100 µl volume) for 6 consecutive days. Mice injected with saline served as controls.

4.2.3.6 Anti-NGF monoclonal antibody

MEDI-578 is a monoclonal antibody, kindly provided by MedImmune, which binds nerve growth factor (NGF) and prevents it from signaling onto its receptors. In **Study II**, MEDI-578 (3 mg/kg) was administered by s.c. injections every 6 days and PBS was used as vehicle control.

4.2.4 Arthritis score

The degree of joint inflammation in the fore and hind paws was assessed daily by a blinded researcher, as previously described (382). A score of 1 point was given for each inflamed toe

or knuckle, and if the ankle/wrist was inflamed, 2.5 or 5 points were given, resulting in a maximum arthritis score of 15 points per paw and 60 points per mouse.

4.2.5 Assessment of pain-like behavior

In **Studies I-IV** mechanical and/or cold hypersensitivity were assessed as a measure of evoked pain-like behaviors. Additionally, in **Study IV** mice were subjected to paw pressure test and grip strength test (performed by co-authors) and their locomotion was monitored. Before any nociceptive testing, mice were acclimatized to the experimental room for 10-15 min. Animals were randomized between cages and the experimenters were blinded to the treatment groups.

4.2.5.1 Mechanical hypersensitivity

Paw mechanical hypersensitivity in the hind paws was assessed using von Frey OptiHair filaments (Marstock) in **Studies I-IV**. Before each baseline testing (3-5 measurements) mice were habituated to the experimental environment (single plexiglass cubicles placed on mesh platform). On the indicated testing days, they were acclimatized to the testing apparatus for 30-60 min prior to the measurements. Filaments with logarithmically incremental stiffness of 0.5, 1, 2, 4, 8, 16, 32 mN (converted to 0.051g, 0.102g, 0.204g, 0.408g, 0.815g, 1.63g and 3.26g respectively) were applied to the plantar surface of the paw and held for 2-3 seconds until a brisk withdrawal of the paw was observed. A cutoff of 4 g was applied in order to avoid tissue damage. The filaments were applied on the right and left hind paw alternatively, with a 5 min interval between stimuli. The Dixon up-down method (383) was used to calculate the 50% withdrawal thresholds (the thresholds at which there was a 50% probability of paw withdrawal). Experiments done by collaborators in University Clermont-Auvergne (France) were performed using Bioseb filaments (0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4g), which is reflected in the different y-axis values, however all calculations and testing were done in a similar fashion. Data is presented in grams or as percentage change to individual baseline values. For certain experiments mechanical hypersensitivity is presented as hyperalgesic index (HI), which is calculated from the area between extrapolated baseline and the time-response curve following certain treatment. Increasing HI index reflects increasing mechanical hypersensitivity.

4.2.5.2 Cold allodynia

Cold thermal sensitivity was assessed using acetone drop method (384) in **Study II and III**. Mice were placed in the same testing device as used for detection of mechanical hypersensitivity. After habituation, a 1 ml syringe was used to gently apply a drop of acetone

to the plantar surface of the hind paw and the duration of the nocifensive behavior (lifting, shaking, biting, rubbing, and licking the paw) was recorded. Absence or delay of these responses was considered as anti-nociceptive effect. The test was repeated three times on each paw and the average was calculated.

4.2.5.3 Locomotion

In **Study IV** locomotor activity was assessed using Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments) cages for 48 h. Mice were single-housed and habituated to the cages for 24 h. The locomotion of the mouse in x-y plane was assessed by automated recording of the beam breaks (counts) in 20 min bins over a 24 h period. The night phase (peak activity phase) was further subdivided in to 4 h bins from 18:00–6:00 hours.

4.2.5.4 Randall-Selitto pressure test

Randall-Selitto test was performed using Analgesy-Meter (Ugo-Basile) to measure the nociceptive withdrawal thresholds of the paw, in **Study IV**. Briefly, mouse was lightly immobilized and a constant, increasing mechanical force was applied to the dorsal surface of the paw using a blunt, conical probe, until a withdrawal response was observed. The maximum force applied was limited to 150 g to avoid tissue damage. Similarly, a thigh-pressure test was performed where increasing pressure was applied to the inner thigh of the mouse. The force in grams at which the mouse withdrew its leg was noted as nociceptive threshold.

4.2.5.5 Grip strength test

Forelimb grip strength test was performed in **Study IV** using a grip strength meter (Ugo Basile) positioned horizontally to the mouse. Animals were habituated to the apparatus twice before the start of the study. Mice were held by the base of the tail and allowed to grab the horizontal bar with their front paws, and then pulled backwards. The measurement of maximum force applied is given once the grip is released. Each mouse was tested at least 2 times and a mean force value was used.

4.2.6 Ethical considerations

My PhD project is solidly based in animal research and would not be possible without the use of laboratory mice. Thus, all the work included in this thesis has been approved by ethical committees and has complied with the ARRIVE and IASP guidelines previously mentioned. The Russell and Burch 3R principle (Replacement, Reduction, Refinement) of humane experimental technique has been thoroughly discussed and implemented in my PhD work.

Replacement: Despite our will to replace the use of animals with other research assays, it has been impossible for this particular thesis work. Although significant effort has been put in our group to find an *in vitro* replacement system for the use of animals for primary cell culture, we were unsuccessful in creating a cell line that could mimic the physiological properties of sensory neurons derived from animals.

Reduction: During planning of each animal experiment substantial amount of time was spent discussing the relevance of the research questions, outcomes and the number of animals that would provide statistically significant answers. The general rule applied to this thesis work was that no more than 10 mice should be used per experimental group. However, if this number was not enough to detect small changes in experimental parameters more mice were included, following a discussion whether it was ethically justified and if it will help us further develop our understanding of studied pain mechanisms. Additionally, extraordinary measures were taken to fully use and/or reuse the tissues collected from each mouse, thereby reducing the number of animals needed.

Furthermore, our lab focuses on the translational aspects of chronic pain research and we have collaborated with clinicians from the rheumatology and pain fields, with the belief that animal research should be done in disease-relevant models. Over the last decades several pain models have been developed, however many of them failed in the process of translating the findings to the clinic. Although, no single animal model will recapitulate all aspects of human pathology, using clinically informed models has a greater chance of identifying novel therapeutics. Important to note is that by conducting research in well-characterized, disease-relevant models we can reduce the number of laboratory animals and focus our efforts on disease-specific experimental studies and, by that, make the most out of the unfortunately inevitable animal research.

Refinement: Carrying out behavioral experiments requires a good understanding and firm control over the general wellbeing of the animals. Throughout this thesis work, it has been extremely important to maintain close contact with the animal technicians and veterinarians to communicate the needs and concerns arounds animal welfare. Furthermore, in order to reduce stress among animals, enriched environment and stable living conditions were constantly monitored. Moreover, once the study started, researchers closely followed the veterinary guidelines and sacrificed the animals as soon as they have reached humane endpoints to minimize their suffering. Additionally, all the behavioral tests that were used allowed the animals to withdraw from the painful stimulus as soon as they perceived it as uncomfortable.

With this approach we have minimized the pain to a level necessary only for the induction of the disease model.

4.3 CELL CULTURE

4.3.1 Mouse osteoclast culture

For **Study I** primary osteoclast cultures were generated from mononuclear hematopoietic cell population isolated from mouse tibial and femoral bones. Following isolation, cells were centrifuged at 300g for 5 min and resuspended in Eagle's Minimum Essential Medium (α-MEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 50 U/ml penicillin and 50 μg/ml streptomycin and seeded onto a T75 flask. Cells were incubated at 37°C and 5% CO2 overnight to allow adherence of stromal cells. Next day, non-adherent cells were removed and harvested by centrifugation for 7 min at 300g. Finally, cells were resuspended in (α-MEM) supplemented with 30 ng/ml macrophage colony - stimulating factor (M-CSF, R&D Systems) and 50 ng/ml receptor activator of nuclear-kappa B ligand (RANKL, R&D Systems) and seeded onto 96-well plate at density 10^6 cells/ml and cultured for 5 days at 37°C and 5% CO2. Human antibodies (1103:01B02, 1325:01B09 and 1276:01G09) were added to the culture at a concentration of 5 μg/ml on the day of seeding and on day 4, during media change. At the end of experiment (day 5) cells were fixed with 4% paraformaldehyde (PFA), washed with 0.01M PBS and TRAP stained using leukocyte acid phosphatase kit (Sigma-Aldrich). The number of multinucleated cells (1-3, 3-9, >9 nuclei) were counted and averaged for each condition (5 wells/condition). Observations have been repeated in three independent experiments.

4.3.2 Human osteoclast culture

For **Study II**, CD14$^+$ monocytes were purified from anonymous female donors (n=3, age >50) by centrifugation through Ficoll-paque (GE Healthare) and subsequently isolated by immunomagnetic separation following the procedure previously described (385,386). Briefly, CD14+ cells were purified using BD IMag Anti-Human CD14 Magnetic Particles – DM (BD Biosciences) and seeded at a density of 5 x 10^6 per T75 flask in α-MEM culture medium, following differentiation into mature osteoclasts over 9 days with 25 ng/mL M-CSF and 25 ng/mL RANKL. Osteoclasts were considered mature when the number of nuclei per cell was between 4-8.

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Mature osteoclasts were harvested by accutase treatment and resuspended in culture media with 25 ng/mL M-CSF and 25 ng/mL RANKL. The viability of detached cells was monitored with trypan blue staining prior to reseeding. Resuspended cells were seeded in 96-well plates on 0.4 mm thick bovine cortical bone slices at a density of 50,000 cells per bone slice. After 60 min incubation at 37°C and 5% CO_2 in a humidified atmosphere, the vehicle control (PBS) or human 1325:04C03 (1 μg/ml) were added to the mature osteoclasts. Five replicates were prepared per condition. Treated cells were incubated for three days at 37°C and 5% CO_2. After incubation, conditioned media from each well was stored at – 20°C. Resorption was visualized with toluidine blue staining as previously described (387) and analyzed by light microscopy to determine the percentage of eroded surface and the number of resorption events. All quantifications were performed blinded.

Conditioned supernatant (20 μl) was mixed with 80 μl TRAcP solution buffer (1 M acetate, 0.5% Triton X-100, 1 M NaCl, 10 mM EDTA (pH=5.5), 8.8 mg/mL L-ascorbic acid, 46 mg/mL disodium tartrate and 18 mg/mL 4-nitrophenylphosphate) and incubated at 37°C for 15 min. The reaction was stopped with 0.3 N NaOH and measured by fluorescence intensity using a microplate reader at an excitation and emission wavelength of 400/645 nm.

4.3.3 Osteoblast culture

For **Study II**, human osteosarcoma cells (Saos-2, Merck), which display osteoblastic features, were resuscitated according to the manufacturer's instructions and cultured for 2 passages in basic culture medium (McCoy's 5A Medium, Sigma), supplemented with 10% FBS (Gibco) and 100 U/mL Penicillin-Streptomycin (Gibco), in 3 T75 flasks (Sarstedt). Cells were harvested by removing the medium, washing once with 1X PBS and incubating for 5 min with 3 mL Trypsin-EDTA (Sigma) solution to facilitate detaching. Trypsinization was stopped by adding 5 mL basic culture medium to the detached cells. The cell suspensions were pooled in a 50 mL falcon tube and centrifuged at 4 °C and 300 RCF for 5 min. Cells were resuspended in 8 mL of medium, counted using a Burker-Turk deep hemocytometer and seeded into 24-well plate or 8-well chamber slide. After reaching confluency Saos-2 cells were differentiated towards an osteoblast phenotype by replacing the basic medium with osteoblast medium supplemented with 100 nmol/L Dexamethasone, 50 μmol/L 2-Phospho-L-ascorbic acid trisodium salt and 10 μmol/L β- Glycerol phosphate disodium salt pentahydrate (all from Sigma), every 2 days. On day 7 or 21 of osteogenic differentiation cells were stimulated for 24 h with different stimuli (see **Study II**). The next day the supernatant was collected and stored at -80°C until analysis.

4.3.4 Mouse macrophage culture

For **Study III,** primary macrophage culture was generated from bone marrow cells isolated from mouse tibia and femur. Cells were cultured in low adherence flasks in DMEM (ThermoFischer) complemented with 10% FBS, 2 mM GlutaMAX (ThermoFischer), 50 U/mL penicillin, 50 μg/mL streptomycin, and 10 ng/mL M-CSF in a humidified incubator at 37°C with 5% C02. Upon reaching 70-85% confluency, cells were dissociated and seeded onto 8-well Nunc™ Lab-Tek™ II CC2 chamber slide (ThermoScientific) at a concentration of $1x10^5$/mL and placed in the incubator overnight. After 24 h of incubation, cells were stimulated with 100 μg/mL of human 1325:01B09 or 1276:01G09 for 1 h in starved (0.1% FBS) or non-starved conditions (10% FBS).

4.3.5 Human macrophage culture

For **Study II**, mononuclear cells were isolated from buffy coats of healthy blood donors by Ficoll centrifugation (GE Healthcare), which was followed by monocyte separation using anti-CD14–conjugated microbeads. Monocytes were cultured at a concentration of 5×10^5 cells/ml in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (all from Sigma Aldrich) in the presence of 25 ng/ml M-CSF (Peprotech), with new medium (50%) and cytokines added at day 3. After 3 days, cells were stimulated with DMEM supplemented with mAbs, chemokines or lipopolysaccharide (LPS) for 24 h (see **Study II**). Next day, the supernatant was collected and stored at -80°C until analysis.

4.3.6 Fibroblast-like synoviocytes culture

Human fibroblast-like synoviocytes were isolated from synovial tissues (obtained from knee or hip orthopedic surgery) through enzymatic digestion using 4 mg/ml Collagenase A and 0.1 mg/ml DNase I (Roche). The dissociated cells were cultured in DMEM complemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Non-adherent cells were removed after overnight incubation and new medium was added. The cells were detached with Trypsin/EDTA (Sigma Aldrich) and split into new medium 1:2 when reaching approximately 80% confluence. Cells from passage 11 were used in the experiments. Cells were resuspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine and seeded at a density of 5×10^4 cells per well in a 96-well plate. Two days later when cells reached 80% confluence, half of the plate was starved for 2 h by replacing the medium with DMEM without supplements. Next,

cells were stimulated for 24 hours with medium containing several stimulants (see **Study II** for details). After 24 hours, the supernatant was collected and stored at -80 °C until analysis.

4.3.7 Neutrophil culture

For **Study II**, neutrophil granulocytes were isolated at room temperature from peripheral blood of healthy individuals using dextran sedimentation and Ficoll-density gradient centrifugation, followed by lysis of red blood cells in 0.2% NaCl solution. The remaining cells were filtered through 70 mm cell-strainer, washed 3 times in PBS, and resuspended in RPMI-1640 medium supplemented with glutamine and 2% FBS at a concentration of $5x10^5$ cells/mL. Cells were seeded in a 96-well plate (5×10^5 cells/well) and incubated for 24 h with mAbs, recombinant human IL-8 or a calcium ionophore (see **Study II** for details). Next day, supernatant was collected and stored at -80 °C until analysis.

4.3.8 Dorsal root ganglia (DRG) culture

For **Study III and IV** primary neuronal and satellite glial cell (SGC) enriched/ neuron-depleted cultures were generated. Briefly, C1-L6 mouse DRGs were dissected and kept in cold PBS until enzymatic digestion using papain (Worthington, 0.8 mg/mL; 30 min at 37°C) followed by collagenase I (Worthington, 12 mg/mL) and dispase II (Sigma Aldrich, 14 mg/mL; 30 min at 37°C). Cells were then gently triturated to form a single-cell suspension in Ham's F-12 Nutrient Mixture medium (Gibco) supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin (all from Gibco). SGC-enriched/neuron-depleted cultures were obtained by seeding the cell suspension onto uncoated glass Nunc^{TM} Lab-Tek^{TM} chamber slides and removing the media with nonadherent cells (including neurons) 1.5 h post-seeding. DRG cultures for neuronal staining were seeded onto 8-well Nunc^{TM} Lab-Tek^{TM} II CC2 chamber slides (ThermoScientific). Cells were maintained at 37°C in 5% CO2 atmosphere and allowed to recover for approximately 20 h before ICC. In order to stain the cells in non-permeabilized conditions, cells were washed and stimulated with 100 μg/mL of human 1325:01B09 or 1276:01G09 IgG in media containing 10% FBS and 50 U/mL penicillin, 50 μg/mL streptomycin for 3 h in humidified incubator.

4.3.9 Electrophysiology and calcium imaging

Electrophysiology and calcium imaging experiments for both **Study I** and **IV** were performed by co-authors of the papers and will be only shortly described below. The outcomes of these experiments will not be discussed in detail in this thesis, for more information please see **Study I** and **IV**.

For **Study I** patch clamp recording was performed in HEK293 cells transfected with ASIC3, to investigate if B02/B09 antibodies can directly activate and/or potentiate ASIC3 currents.

Electrophysiological recordings from skin-nerve preparations were performed for **Study IV** in order to examine if IgG from fibromyalgia patients sensitizes peripheral nociceptors. Furthermore, calcium imaging on DRG neurons was performed to assess if FMS IgG directly activates sensory neurons.

4.4 IMAGING ANALYSES

4.4.1 2-DeoxyGlucosone 750 imaging

For **Study I** glucose uptake as a surrogate of inflammation was measured *in vivo* using IVIS spectrum imaging system (Perkin Elmer). Mice were injected i.v. with 10 nmol/100 µl of XenoLight RedIJect 2-deoxyglucosone (DG)-750 (Perkin Elmer), a fluorescent probe that specifically accumulates in regions with enhanced glucose uptake. Three hours later imaging was performed under isoflurane anesthesia and using ex/em = 745/820 nm bandpass filter. Quantification analysis was performed with Living Image Software using ROI delineated on hind paws (ankle joint). Results are presented as the average of both hind paws of total counts of radiance efficiency (radiance (photons per second per square centimeter per steradian) per incident excitation power (microwatt per square cm)).

4.4.2 Cathepsin K imaging

For **Study I and II** bone remodeling was assessed using cathepsin K *in vivo* imaging. Mice were injected i.v. with 2 nmol/100 µl Cat K 680 FAST^{TM} (Perkin Elmer) fluorescent imaging agent, which localizes to the sites of increased bone resorption and osteoclast activity. Six hours later, image acquisition was performed using IVIS spectrum imaging system (Perkin Elmer) under isoflurane anesthesia, using ex/em=675/720 nm bandpass filter. Quantification was performed as described form 2-DG imaging.

4.4.3 Scintigraphic imaging

To evaluate bone and cartilage remodeling in **Study I**, *in vivo* scintigraphic imaging was performed. Mice were i.v. injected with either 25 MBq of ^{99m}Tc-NTP 15-5 to visualize cartilage or with 10 MBq of ^{99m}Tc-HMDP to visualize bone. Imaging was performed under isoflurane anesthesia 30 min post ^{99m}Tc-NTP 15-5 injection and 2.5 hours post ^{99m}Tc-HMDP injection using gamma imager for small animals (γImager, Biospace). Quantitative analysis of both

scintigrams was performed with Gammavision+, with ROIs delineated over the hind paws. Results are presented as the average of both hind paws of the total counts in cpm in the ROI.

4.4.4 Micro-computer tomography imaging

For **Study I and II** bone erosion was evaluated using micro-computer tomography imaging (micro-CT). Prior to imaging, hind legs were harvested from euthanized mice, post-fixed in 4% PFA for 48 h and stored in 0.01 M PBS until use. Proximal tibia, calcaneus and thalus were scanned using Skyscan 1272 system (Bruker) at a 10 μm voxel size, 60 kVp, 166 μA and integration time 627 ms. Images were reconstructed using NRecon software (Bruker) and analyzed using CT analyzer program (Bruker). The parameters used for the trabecular bone were: bone mineral density (BMD), trabecular bone volume ratio (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N). Finally for cortical bone, cortical thickness (Ct.Th) was obtained.

4.5 HISTOLOGICAL AND MOLECULAR ANALYSES

4.5.1 Joint histology

For **Study I and II**, hind ankle joints were post-fixed in 4% PFA for 48 h. Next, tissues were decalcified in 10% EDTA for 4-5 weeks, dehydrated in ethanol and embedded in paraffin. Sagittal sections (5 μm) were cut and stained with hematoxylin and eosin and scored by two blinded investigators on a scale from 0-3, where 0 is normal and 3 is severe synovitis, bone erosion, and/or cartilage destruction.

4.5.2 Immunohistochemistry

For **Study I, II, IV** mice were terminally anesthetized and perfused transcardially with saline followed by 4% PFA. Lumbar DRGs (L3-L5) and hind paw glabrous skin (**Study IV**) were collected, post-fixed in PFA for 24 h, cryoprotected in 20 or 30% sucrose in 0.01 M PBS for 48 h at 4°C and then embedded in freezing tissue medium (OCT). Tissue was sectioned with CryoStar NX70 cryostat and mounted onto SuperFrost Plus slides.

For **Study I**, transverse sections (12 μm) were cut on cryostat and dried for 30 min at room temperature (RT) before staining. Following 3 washes in PBS, sections were blocked with PBS containing 0.2% Triton X-100 and 1% bovine serum albumin (BSA). Slides were then incubated overnight at RT in a humid chamber with primary antibodies diluted in PBS + 0.2% Triton X-100 and 1% BSA (listed in the Table 1). Next day, after 5 washes with PBS, sections

were incubated with appropriate secondary antibodies for 2 h, at RT. Finally, sections were washed in PBS and cover slipped with fluorescent mounting medium. Negative control sections were stained only with secondary antibody and subjected to the same experimental conditions.

For **Study II**, tissue was sectioned at a thickness of 15 μm and dried before staining. Slides were blocked with 0.01M PBS supplemented with 3% normal goat serum (NGS) and 0.3% Triton X-100 for 1 h and then incubated overnight with human 1325:04C03 hIgG1, 1325:01B09 hIgG1 and 1276:01G09 hIgG1 antibodies (see Table 1) and primary anti-GS antibody diluted in PBS with 1% NGS and 0.1% Triton X-100. Next day slides were washed and incubated with fluorescently conjugated antibodies against human IgG and appropriate secondary antibodies for 1.5 hours at RT. Following washing, slides were incubated with NeuroTrace 640/660 Deep-Red Fluorescent Nissl Stain (1:200) for 20 min, washed and counterstained with DAPI for 10 min (1:10.000). Finally, slides were washed, and cover slipped with ProLong Gold mounting media.

For **Study IV**, lumbar DRGs and glabrous skin were sectioned at a thickness of 10 μm. Slides were blocked with PBS supplemented with 0.3% Triton X-100 and 3% NGS or donkey serum, depending on the secondary antibody, followed by incubation with primary antibodies diluted in PBS with 0.1% Triton X-100 and 1% serum. For colocalization studies of human IgG with various cell-type markers the primary antibodies against the cell-type markers were first added, and then following washing, the anti–human IgG antibody was co-incubated with the secondary antibodies against the cell-type marker primary antibodies. Following washing, the slides were incubated with DAPI (1:20.000), washed again, and then cover slipped with Prolong Gold mounting media.

For **Study IV** a human DRG was collected, flash frozen and stored at -80°C until use. Tissue was sectioned at a thickness of 14 μm, fixed for 30 min with cold 4% PFA, washed and blocked with PBS supplemented with 0.3% Triton X-100 and 3% NGS for 1 h. Next, tissue was incubated overnight with 100 μg/ml unconjugated anti-human IgG Fab fragments. Following washing, slides were incubated overnight with pooled FMS or HC IgG (15 μg/ml) diluted in PBS with 1% NGS and 1% Triton X-100. Next day slides were incubated with appropriate secondary antibodies for 2 h. Primary antibodies against NF200 and GFAP were applied for 2 h, following incubation with secondary antibodies, washing, and incubating with DAPI (1:10.000). Finally, slides were cover slipped with Prolong Gold mounting media.

Table 1. List of antibodies used for IHC

Primary Ab	Tissue	Dilution	Vendor/catalog	Study
Guinea pig anti-ASIC3	L3-L5 DRG	1:200	Merck, #AB5927	I
Rabbit anti-GS	L3-L5 DRG	1:500	Abcam, #ab73593	II, III, IV
Rabbit anti-Iba-1	L3-L5 DRG	1:500	Wako, #019-19741	IV
Rabbit anti-GFAP	L3-L5 DRG	1:500	Milipore, #Mab360	IV
Chicken anti-NF200	Human DRG	1:500	Neuromics, #CH22104	IV
Rabbit anti-PGP 9.5	Skin	1:2000	Cedarlane, #CL7756AP	IV
Rat anti-CD31	L3-L5 DRG	1:500	BD Pharmigen, #Ab739	IV
Goat anti-TrkA	L3-L5 DRG	1:100	R&D Biosystems, #AF5479	IV
Human G09/C03/B09 mAbs	L3-L5 DRG	5 µg/ml	-	II

4.5.3 Immunocytochemistry

For **Study III**, immunocytochemistry (ICC) was performed on macrophage and DRG/SGC primary cell cultures. Cells were live incubated in humid incubator with human B09 and G09 mAbs for 3 h. After incubation with antibodies, cells were washed 2x with 0.01 M PBS, fixed with 4% PFA for 10 min, washed and blocked for 20 min with solution containing 5% NGS in PBS. Neurons and SGC were visualized by staining with anti-PGP 9.5 and anti-GS and macrophages with anti-Iba-1 primary antibodies, respectively. Following incubation with primary antibodies, cells were washed and incubated with fluorescently conjugated antibodies against human IgG and appropriate secondary antibodies for 1.5 h, at RT. Next, cells were washed and incubated with DAPI for 10 min (1:5000), washed again and cover slipped with Prolong Gold mounting media.

For **Study IV**, DRG/SGC primary cell cultures were live incubated with fibromyalgia (FMS) or healthy control (HC) pooled IgG diluted in media with 10% FBS and 1x Penicilin-Streptomycin, for 3 h. Cell were then washed with PBS, fixed with 4% PFA for 10 min, washed and blocked with PBS supplemented with 5% NGS. SGCs and neurons were visualized by adding primary antibodies (anti-GS or anti-β-III tubulin, respectively) diluted in the blocking solution to the cells. Next, cells were washed and incubated with fluorescently conjugated antibodies against human IgG and appropriate secondary antibodies. Finally, cells were incubated with Hoechst (1:5000), washed and cover slipped with Prolong Gold mounting media.

Table 2. List of primary antibodies used for ICC

Primary Ab	Cell type	Dilution	Vendor/catalog	Study
Rabbit anti-GS	DRG/SGC culture	1:500	Abcam, #ab73593	IIII, IV
Rabbit anti-PGP 9.5	DRG/SGC culture	1:4000	Promega, #CL7756AP	III
Mouse anti-β-III tubulin	DRG/SGC culture	1:2000	Promega, #G7121	IV
Rabbit anti-Iba-1	Macrophage culture	1:500	Wako, #019-19741	III
FMS/HC IgG	DRG/SGC culture	100 µg/ml	-	IV
Human G09/C03/B09 mAbs	DRG/SGC culture	100 µg/ml	-	III

4.5.4 Confocal imaging

Representative pictures of cell cultures and DRGs were taken using a confocal microscope (Zeiss LSM800) operated by LSM ZEN2012 (Zeiss) software.

4.5.5 Quantitative polymerase chain reaction

Flash frozen tissues (ankle joints, L3-L5 DRGs) were homogenized using TRIzol reagent. Total RNA was extracted followed by cDNA preparation according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using pre-developed hydrolysis probes listed in the Table 3. Data were normalized against housekeeping genes Rplp2 or Hprt. Relative fold changes were calculated using the comparative Ct method (2-ΔΔCt).

Table 3. List of TaqMan primers used in the thesis

Gene	Full name	Taqman primer	Paper
Acp5	Acid phosphatase 5, tartrate resistant	Mm00475698_m1	I, II
Adgre	Adhesion G protein-coupled rec. E1	Mm00802529_m1	III
Aif1	Allograft inflammatory factor	Mm00479862_g1	II, III
Asic1	Acid-sensing ion channel subunit 1	Mm01306001_g1	I
Asic3	Acid-sensing ion channel subunit 3	Mm00805460_m1	I
Bdnf	Brain-derived neurotrophic factor	Mm01334042_m1	III
Cacna2d1	Calcium channel, voltage-dependent, alpha2/delta subunit 1	Mm00486607_m1	III
Calca	Calcitonin gene-related peptide	Mm00801462_m1	III
Clcn7	Chloride channel 7	Mm00442400_m1	I, II

Ctsk	Cathepsin K	Mm00484039_m1	I, II
Ctss	Cathepsin S	Mm01255859_m1	II, III
Csf1	Colony stimulating factor 1	Mm00432686_m1	III
Cxcl1	Chemokine (C-X-C motif) ligand 1	Mm04207460_m1	I, II, III
Cxcl2	Chemokine (C-X-C motif) ligand 1	Mm00436450_m1	I, II, III
Cx3Cl1	Chemokine (C-X3-C motif) ligand 1	Mm00436454_m1	II, III
Cx3Cr1	Chemokine (C-X3-C motif) receptor 1	Mm00438354_m1	II, III
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Mm00516572_m1	I
Gap43	Growth-associated protein 43	Mm00500404_m1	II
Gfap	Glial fibrillary acidic protein	Mm01253033_m1	II
Glul	Glutamate-ammonia ligase (glutamine synthetase)	Mm00725701_s1	III
Hprt	Hypoxanthine guanine phosphoribosyl transferase	Mm03024075_m1	II, III, IV
Il1β	Interleukin-1β	Mm00434228_m1	I, II, III
Il17Ra	Interleukin-17 receptor A	Mm00434214_m1	III
Il33	Interleukin-33	Mm00505403_m1	III
Il6	Interleukin-6	Mm00446190_m1	I, II, III
Itgam	Integrin alpha M	Mm00434455_m1	III
Kcnj10	Potassium inwardly rectifying channel, subfamily J, member 10	Mm00445028_m1	III
Lpar1	Lysophosphatidic acid receptor 1	Mm00439145_m1	I
Lpar2	Lysophosphatidic acid receptor 2	Mm00469562_m1	I
Lpar3	Lysophosphatidic acid receptor 3	Mm00469694_m1	I
Lpar4	Lysophosphatidic acid receptor 4	Mm01228532_m1	I
Lpar5	Lysophosphatidic acid receptor 5	Mm01190818_m1	I
Lpar6	Lysophosphatidic acid receptor 6	Mm00163058_m1	I
Ngf	Nerve growth factor	Mm00443039_m1	II, III
Ngfr	Nerve growth factor receptor	Mm00446296_m1	II
Ntf3	Neurotrophin 3	Mm01182924_m1	II
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	Mm01219406_m1	II, III
Pla2g2a	Phospholipase A2, group IIA	Mm00448160_m1	I
Pla2g5	Phospholipase A2, group V	Mm00448162_m1	I
Pla2g10	Phospholipase A2, group X	Mm01344436_g1	I
Ptgs2	Prostaglandin-endoperoxide synthase 2	Mm00478374_m1	I, II
Tac1	Tachykinin 1	Mm01166996_m1	III

Tcirg1	V-type proton ATPase isoform A3	Mm00469406_m1	I, II
Tnf	Tumor necrosis factor	Mm00443258_m1	I, II, III
Trpm8	Transient receptor potential cation channel, subfamily M, member 8	Mm01299593_m1	III
Trpv1	Transient receptor potential cation channel, subfamily V member 1	Mm01246302_m1	I, III
Rplpl2	Ribosomal protein P2	Mm00782638_s1	I, II, III
S100β	S100 protein, beta polypeptide, neural	Mm00485897_m1	III, IV

4.5.6 Immunoassay

In **Study I**, ELISA assay was established to detect the levels of lipocalin-2 in the tissue. Ankle joints were crushed in liquid nitrogen and homogenized in lysis buffer. Homogenates were centrifuged at 14.000 g for 10 min at 4 °C and supernatants were collected following BCA Protein Assay to determine protein concentrations in cell lysates. Lipocalin-2 (LCN2) was measured using a quantitative sandwich enzyme immunoassay technique according to the manufacturer's protocol (DuoSet, #DY1857, R&D). Samples were processed in duplicates; final values were calculated from the standard curve.

For **Study II**, mesoscale immunoassay (U-PLEX Metabolic Singleplex Assay) was used to assess the presence of β-NGF in the supernatant of human fibroblasts, macrophages, neutrophils and osteoblasts stimulated with 1325:04C03hIgG1 or 1362:01E02 control antibody. Supernatants of unstarved fibroblasts were diluted 2X and supernatant from starved fibroblasts were diluted 4X. Macrophage, neutrophil and osteoblast supernatants were used undiluted. Results are presented as pg/mL and depicted as mean ± standard error of mean (SEM).

For **Study III**, mesoscale was used to determine the levels of CXCL1 in the supernatant of SGC cells. SGCs were stimulated with 1325:01B09 hIgG1 (1 μg/ml) alone or with different LPS concentrations (5-50 ng/ml) for 24 h. Levels of CXCL1 (KC-GRO) were measured in the supernatant according to the manufacturer's instructions, results are presented as pg/mL and depicted as mean ± standard error of mean (SEM).

In **Study IV**, mesoscale (V-Plex Proinflammatory Panel 1) was used to measure the concentrations of cytokines and chemokines in the serum of mice injected with FMS, HC IgG or saline. The concentrations of TNF, IL-6, IL-2, IL-5, IFNγ, IL-10 and CXCL1 were measured according to the manufacturer's instructions. All serum samples were dilute 1:2 and measured as technical duplicates. The lower limit of detection for each analyte was defined as the mean

signal of the blank plus 2.5 standard deviations and the lower limit of quantification was obtained from the manufacturer. Results are presented as pg/mL and depicted as mean ± standard error of mean (SEM).

4.5.7 Fluorescence-activated cell sorting

For **Study III**, fluorescence-activated cell sorting (FACS) was performed on L3-L5 DRGs collected from mice injected with 1325:01B09 hIgG1 (2 mg/mouse) or control (saline). Tissue was gently homogenized and digested at 37°C for 30-40 min with an enzyme cocktail (1 mg collagenase A with 0.5 mg trypsin in 1 ml DMEM, Sigma). Cells were stained with fluorochrome-labelled antibodies listed in the table (Table 4). Samples were acquired by LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FACSDiva software; counting beads were added. Forward scatter was used for all cellular analysis, as a trigger to identify events.

Table 4. List of fluorophores used for FACS in Paper III

Target	Clone	Fluorophore	Vendor/Catalog
F4/80	BM8	FITC	BioLegend/123108
Cd11b	M1/70	PerCPCy5.5	BioLegend/101227(8)
CD45	30-F11	APCeF780	eBioscience/47-0451-82
MHC II	M5/114.15.2	PE-Cy7	BioLegend/107629
Ly6C	AL-21	BV421	BD Bioscience/562727
CD11c	N418	BV785	BioLegend/117336
CD115	AFS98	APC	eBioscience/1277550

4.6 STATISTICAL ANALYSES

Statistical tests were performed using GraphPad Prism 8. Results were analyzed using unpaired two-tailed student's t-test (differences between two groups), one-way analysis of variance, (ANOVA; differences between multiple groups), followed by Bonferroni post-hoc test and two-way ANOVA (for changes between multiple groups compared over time), followed by Bonferroni post-hoc test. All data are presented as mean ± standard error of mean (SEM) and p-values less than 0.05 were considered statistically significant.

5 RESULTS AND DISCUSSION

5.1 STUDY I: CONTRIBUTION OF OSTEOCLASTS AND ASIC 3 SIGNALING TO PAIN-LIKE BEHAVIOR INDUCED BY RA-ASSOCIATED ANTIBODIES

Joint pain (arthralgia) and bone erosion in RA have been classically considered to be the consequences of inflammation and tissue injury. However, several clinical reports show that these two RA features can occur long before the disease onset (291,388,389), suggesting that other non-inflammatory mechanisms are involved. An increasing amount of evidence links pain and bone loss to autoantibodies, which are present in certain cohorts of RA patients and can be detected many years before the disease onset. Our group has previously shown that transfer of polyclonal anti-citrullinated protein antibodies (ACPA) to naïve mice causes pain-like behavior and bone loss without visible signs of inflammation (138,229). To build on the previous work, we have used a combination of two monoclonal antibodies isolated from synovial B cells of RA patients to study the underlying mechanisms of their pronociceptive and erosive properties.

In this study, we have combined an anti-modified protein antibody (1325:01B09 IgG), known to react with citrullinated, acetylated and carbamylated antigens with 1103:01B02 IgG, an antibody with unknown binding properties. Although at the time when our work on this project has started, we considered B02 mAb to bind citrullinated antigens, extensive analysis using optimized assays demonstrated that it does not have any citrulline reactivity (239). Previously we have demonstrated that B02 mAb binds and stimulates osteoclasts *in vitro* and induces pain-like behavior and bone erosion when injected into mice (138,229). In contrast, B09 mAb was shown to lack stimulatory effects on osteoclasts, but instead it binds nuclear antigens in activated neutrophils (390) and stimulates migration of stressed fibroblast-like-synoviocytes (298). Thus, despite the setback associated with unknown antigenicity of B02 mAb, we still considered this antibody combination worth studying due to the differential effects of B02 and B09 mAbs on cells in the joint.

To compare the pronociceptive effects of B02 and B09 mAbs, we have injected them i.v. alone (1 mg/mouse) or in combination (2 mg/mouse) into naïve female mice and monitored the arthritis scores and mechanical thresholds. We have observed that mice injected with B09 mAb displayed a long-lasting mechanical hypersensitivity, until day 21 post-injection, whereas B02 mAb induced only a short-lasting mechanical hypersensitivity detectable at days 7 and 10 post-injection (Study I, Figure 1). Interestingly, combining B02 and B09 enhanced and prolonged

mechanical hypersensitivity, compared to injection of these antibodies alone, suggesting a potentiation of B09 pronociceptive effect, when combined with B02 mAb.
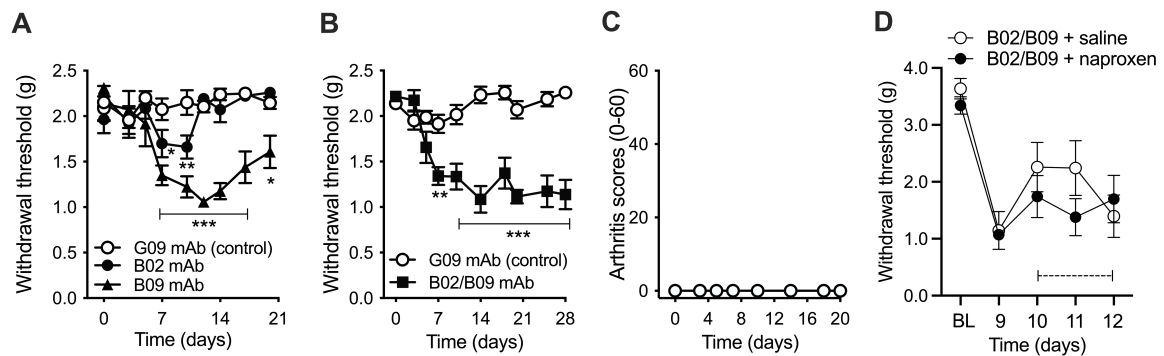
Figure 6. Monoclonal antibodies from RA patients act in synergy to prolong pain-like behavior, which is not associated with overt inflammation. **(A)** Single i.v. injection of B02 or B09 mAb induces transient mechanical hypersensitivity, as compared to G09 control mAb. **(B)** Combining B02 and B09 leads to long-lasting pain-like behavior, detectable 28 days post-injection. **(C)** Neither of the mAbs, injected alone or in combination induces visible joint inflammation, measured as arthritis score (0-60). **(D)** Pain-like behavior induced by B02/B09 is not reversed by repeated treatment with naproxen (50 mg/kg). Data presented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001, n=6-11/group. Adapted from **Study I**, Figure 1 and 3.

While none of the antibodies, injected alone or in combination induced any visual signs of inflammation in the joint, we have performed several *in vivo* and *in vitro* analyses to assess the potential involvement of subclinical inflammation to B02/B09-induced hypersensitivity. Although *in vivo* imaging, lipocalin levels and histological assessment of the ankle joint did not suggest an overt inflammatory process, we found a modest increase in the expression of proinflammatory cytokines (*Tnf, Il-6* and *Ptgs2*) in the joints of B02/B09 mice. As a comparison, we have evaluated the expression of six proinflammatory cytokines in the ankle joints from the inflammatory phase of CAIA model and found them all to be upregulated with a much higher magnitude than in B02/B09 joints (Study I, Figure 2). These data suggested that B02/B09-induced pain-like behavior is accompanied only by low-grade subclinical joint inflammation that cannot be detected by means of visual inspection. Despite the possible contribution of subclinical inflammation to pain-like behavior in this model, we excluded it as the sole driver of mechanical hypersensitivity, as we found that treatment with naproxen and paracetamol did not reverse pain-like behaviors in B02/B09-injected mice (Study I, Figure 3). Thus, we concluded that B02/B09-induced mechanical hypersensitivity is most probably driven by other mechanisms.

We next tested if B02 and B09, alone or in combination, lead to changes in bone metabolism and cause bone erosion. Using *in vivo* scintigraphy, micro-CT and osteoclast cultures we have demonstrated that B02/B09 combination stimulates osteoclast activity *in vitro*, rapidly increases bone metabolism and leads to significant bone erosion *in vivo* (Study I, Figure 4). Importantly, although B02 alone did not cause significant bone erosion *in vivo*, it stimulated

osteoclast differentiation *in vitro*, confirming previous findings done in human osteoclast cultures (229).

In order to assess if bone erosion is coupled to B02/B09-induced hypersensitivity, we have tested the antinociceptive effects of two osteoclast inhibitors: zoledronate and cathepsin K inhibitor T06. Systemic treatment with either of these inhibitors successfully prevented the development of pain-like behaviors following B02/B09 injection (Study I, Figure 5), pointing to a direct relationship between osteoclast activity and mechanical hypersensitivity in B02/B09 mice.
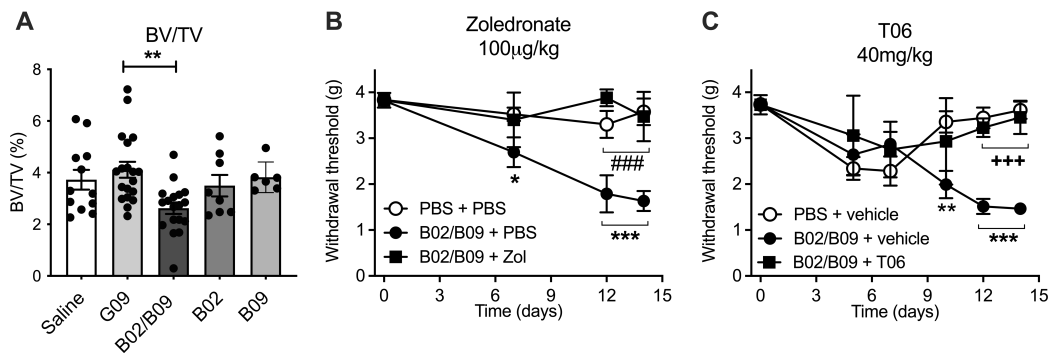
Figure 7. B02/B09 mAbs induce bone erosion and osteoclast inhibitors prevent B02/B09-induced mechanical hypersensitivity. **(A)** Micro-CT analysis demonstrates that B02/B09 mAbs reduces trabecular bone volume in the proximal tibia, compared to G09 control Ab, n=6-19/group. **(B)** Treatment with zoledronate (100 μg/kg, n=6/group) or **(C)** T06 (40 mg/kg, n=6-8/group) prevents the development of B02/B09-induced pain-like behavior in mice. Data presented as mean±SEM, *p<0.05, **p<0.01, *** or ### or +++ p<0.001. Adapted from **Study I**, Figure 4 and 5.

Actively resorbing osteoclasts release protons through the action of V-ATPase to facilitate degradation of bone mineral matrix. As mentioned in the introduction, nociceptors express acid-sensing ion channels such as ASIC3 and thus can detect acidification of local bone environment. In order to assess if ASIC3 is involved in B02/B09-induced mechanical hypersensitivity, we have targeted ASIC3 pharmacologically using APETx2, a selective ASIC3 inhibitor, as well as genetically using ASIC3-deficient mice. We found that blockade and/or deletion of ASIC3 protect mice from B02/B09-induced mechanical hypersensitivity (Study I, Figure 6). We also observed that injection of B02/B09 increases the percentage of ASIC3 positive neurons in the L3-L5 DRGs, as well as enhances the expression of *Asic3* in the ankle joints, compared to PBS-injected controls. Importantly, in order to confirm that ASIC3 is activated by protons released from B02/B09-activated osteoclasts, we have used bafilomycin A1 to block the action of V-ATPase. Intriguingly, treatment with this inhibitor did not attenuate B02/B09-induced pain-like behavior, suggesting that mechanical hypersensitivity is not dependent on acidification, and that other osteoclast-derived ligands might activate ASIC3.
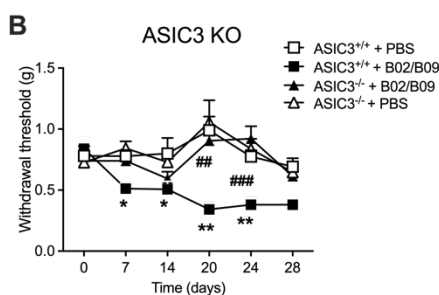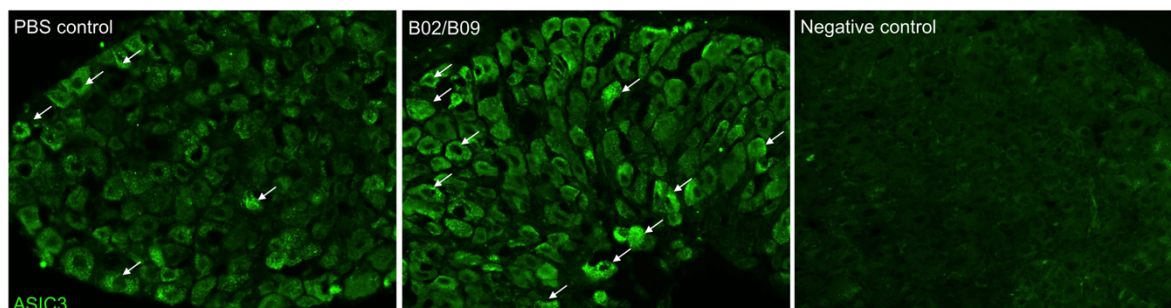
Figure 8. B02/B09-induced mechanical hypersensitivity is dependent on ASIC3. **(A)** Intravenous injection of B02/B09 increases the percentage of ASIC3-positive neurons in the L3-L5 DRGs, as compared to PBS control, scale bar = 100 μm. Arrows point to representative cells with positive ASIC3 staining. **(B)** ASIC3 knock-out mice are protected from B02/B09-induced pain-like behavior, in comparison with WT animals, n=26-29 mice/group until day 14 and n=9-19/group until day 28. Data presented as mean±SEM, *p<0.05, ** or ## p<0.01, ### p<0.001. Adapted from **Study I**, Figure 6.

Extracellular protons are considered the dominant endogenous triggers of ASIC3 activation. However, certain lipids such as AA or LPC were shown to activate ASIC3 at physiological pH (48). Moreover, increased levels of LPC were found in the knee synovial fluid of RA patients (48,391) and LPC injection into mice was shown to induce pain-like behaviors (391). Thus, we sought to investigate if B02/B09-induced pain-like behavior is accompanied by increased LPC levels that could sensitize ASIC3. Mass-spectrometry analysis has shown that, out of several LPC species that were measured, LPC 16:0 was the only one significantly increased in the bone marrow of B02/B09 mice, compared to controls (Study I, Figure 8). We then assessed if elevated levels of LPC 16:0 are associated with increased expression of sPLA$_2$, enzyme responsible for LPC production. We found that two out of three sPLA$_2$ isoforms (*Pla2g2a*, *Pla2g5*) were upregulated in B02/B09 ankle joints and, importantly, reduced with zoledronate treatment (Study I, Figure 8). Since sPLA$_2$ was shown to be involved in inflammation, we assessed its expression in the joints from CAIA inflammatory phase and found no significant upregulation of either of the two sPLA$_2$ isoforms. These findings suggest that sPLA$_2$ upregulation in B02/B09 joints is unlikely to be due to an ongoing inflammatory process.

Finally, to investigate if B02/B09-induced mechanical hypersensitivity is dependent on sPLA$_2$ activity, we treated B02/B09-injected mice with varespladib, an inhibitor of all three isoforms of sPLA$_2$. We found that repeated injections of the inhibitor significantly reversed both

mechanical hypersensitivity and bone erosion in the treated group (Study I, Figure 8). This indicates that sPLA$_2$ is involved in autoantibody-driven bone loss and pain-like behavior in this model.
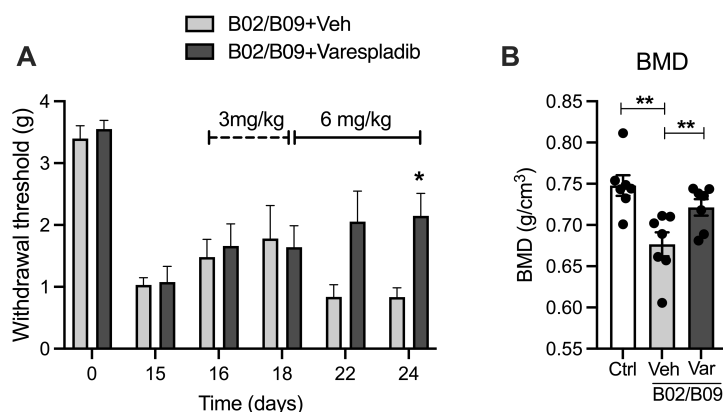
Figure 9. sPLA$_2$ inhibitor reverses B02/B09-induced mechanical hypersensitivity and bone erosion. **(A)** Dose-escalation treatment with varespladib (3 mg/kg on days 16-18, 6 mg/kg on days 19-24 post-B02/B09 administration), reverses pain-like behavior induced by B02/B09, compared to mice injected with vehicle (20% DMSO in PBS). **(B)** Varespladib normalizes bone mineral density of the thalus, reversing B02/B09-induced bone erosion. Data presented as mean±SEM, n=6-7/group, **p<0.01. Adapted from **Study I**, Figure 8.

Taken together, our data suggest a novel mechanism through which RA-associated monoclonal antibodies generate pain-like behavior in mice. We found that B02/B09-induced mechanical hypersensitivity does not depend on classical inflammatory processes and cannot be reversed with NSAIDs or paracetamol. Instead, we have shown that B02/B09-induced pain-like behavior is coupled to osteoclast and ASIC3 activity. These findings are in line with previous preclinical studies which demonstrated that targeting osteoclasts and ASIC3 signaling can act anti-nociceptive in several models of bone-related pain (136,139,170,392,393). Intriguingly, we have identified sPLA$_2$ and LPC 16:0 as possible links between increased bone erosion and ASIC3 sensitization. Injections of both sPLA$_2$ and LPC 16:0 were shown to induce hypersensitivity in mice, suggesting their direct role as pain-triggering factors (163,164,391). While the exact source of sPLA$_2$ remains to be identified in this model, it has been previously shown that osteoclasts, chondrocytes and fibroblasts-like synoviocytes can produce sPLA$_2$ (162,394–396). Hence, we hypothesize that either B02 mAb activates osteoclasts and/or B09 mAb stimulates fibroblasts to release sPLA$_2$, which catalyzes LPC 16:0 production, and ultimately leads to ASIC3 activation and sensitization. From a translational point of view, our work can help understand the mechanisms that drive arthralgia in the pre-clinical phase of RA, where joint inflammation is not yet detected, but patients already have pain and bone erosion.

5.2 STUDY II: NGF MEDIATES PAIN-LIKE BEHAVIOR INDUCED BY OSTEOCLAST-ACTIVATING MONOCLONAL ACPA

Increasing amount of evidence points to the role of anti-citrullinated protein antibodies (ACPA) in mediating the pathogenesis and development of RA. Previous studies from our group have demonstrated the relationship between the presence of ACPA IgG and pain-like behavior and bone erosion in mice. While the exact mechanisms remain to be elucidated, through *in vitro* studies we have shown that ACPA IgG drives the release of CXCL1 from human and mouse osteoclasts, which then further stimulates them in an autocrine manner (138,229). Moreover, intra-articular injection of CXCL1 was shown to be pronociceptive in mice and treatment with CXCR1 receptor antagonist reparixin prevented ACPA-induced bone loss and hypersensitivity. While these studies provide evidence for the functional properties of ACPAs, due to the use of polyclonal IgG they do not fully delineate the complex mechanisms that drive ACPA-induced bone erosion and pain-like behavior. Thus, it is important to study single monoclonal ACPAs to fully understand their pathological properties.

The aim of this study was to explore the mechanisms through which monoclonal 1325:04C03 IgG, previously shown to stimulate osteoclast differentiation and increase artificial bone erosion *in vitro* (238,298), induces pain-like behavior in mice.

Mice injected i.v. with 2 mg of C03 mAb (human or murine) developed significant mechanical hypersensitivity and displayed cold allodynia 9 days post-injection, as compared to control mice. Importantly, these evoked pain-like behaviors were not accompanied by any visual signs of joint inflammation, measured as arthritis score (Study II, Figure I). In addition, local injection of C03 mAb into the ankle joint resulted in mechanical hypersensitivity detectable 6- and 24 hours post-injection as compared to non-injected paw. This demonstrates that C03 mAb evokes mechanical hypersensitivity both when injected systemically or locally in the joint.

While we have detected increased levels of mRNA for several proinflammatory cytokines in the ankle joints of C03-injected mice, treatment with two NSAIDs (diclofenac and naproxen) did not alleviate C03-induced mechanical hypersensitivity (Study II, Figure I). Together with the lack of histological signs of inflammation in the ankle joint, like synovial hyperplasia or cell infiltration, these data suggest that C03-induced pain-like behavior is not driven by inflammation.
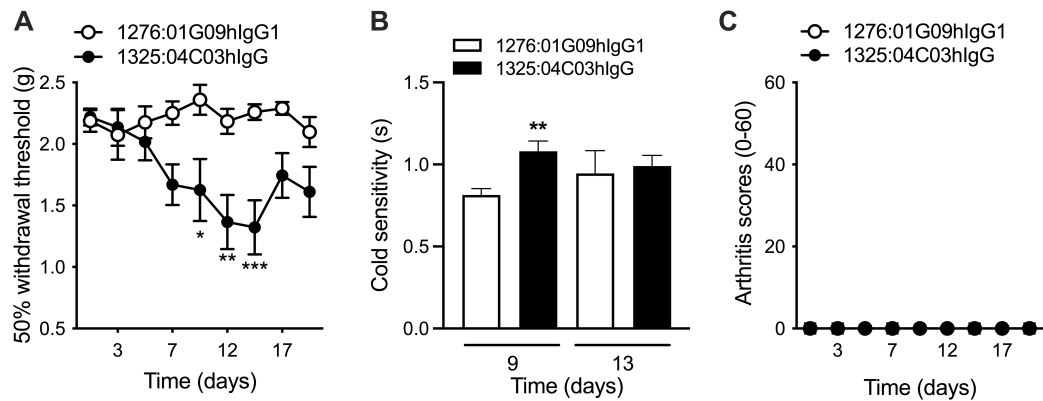
Figure 10. Monoclonal C03 Ab induces mechanical and cold hypersensitivity in the absence of joint inflammation. **(A)** Single i.v. injection of human C03 (2 mg/mouse) induces mechanical hypersensitivity and **(B)** cold sensitivity, compared to human control antibody G09 (2 mg/mouse). **(C)** Human C03 does not cause visual signs of inflammation, compared to control and measured as arthritis score (0-60). Data presented as mean±SEM, n=6-16/group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Adapted from **Study II**, Figure 1.

Previous studies using human osteoclasts demonstrated that C03 mAb stimulates osteoclastogenesis *in vitro* (238), however its effects on bone metabolism *in vivo* were so far not investigated. Surprisingly, using *in vivo* cathepsin K imaging and micro-CT in the proximal tibia and calcaneus we concluded that C03 mAb does not alter bone metabolism or bone microarchitecture (Study II, Figure 2). However, despite the lack of macroscopic changes in bone morphology we assessed the expression of osteoclast-related genes in the ankle joints of C03-injected mice and found some of them to be upregulated, compared to control joints. In order to understand if C03 mAb directly activates osteoclasts, we have established human osteoclasts cultures and incubated them with the antibody. While C03 mAb stimulated osteoclastogenesis on day 3 and 5 (measured as increased TRAcP activity), it did not have any effect on day 7, suggesting only a transient effect on differentiation. Furthermore, several osteoclast activity parameters were measured to assess the effect of C03 mAb on bone-eroding capacity of the cells, but neither of them has demonstrated a potentiating effect of C03 mAb.

Although our data does not point to increased erosive activity of osteoclasts in response to C03 mAb, an increasing number of studies shows that osteoclasts can display alternative modes of activation and release factors which are not directly associated with bone erosion, such as IL-8, netrin-1 or semaphorin 4D (170,229,397). In order to ascertain that osteoclasts are important for C03-induced mechanical hypersensitivity, we treated C03-injected mice with zoledronate and found that it partially prevents mechanical allodynia in mice (Study II, Figure 2). Since C03 mAb administration led to enhanced expression of *Cxcl1* in the ankle joints, we next assessed the anti-nociceptive effects of reparixin, a CXCR1/2 antagonist. Similarly to what we saw with polyclonal ACPA (138), blocking CXCL1/2 signaling prevented the development of mechanical hypersensitivity in C03-injected mice. This indicates that while osteoclast activity

is at least partially important for C03-induced pain-like behavior other osteoclast-derived pronociceptive factors, like CXCL1/2, can contribute to hypersensitivity.
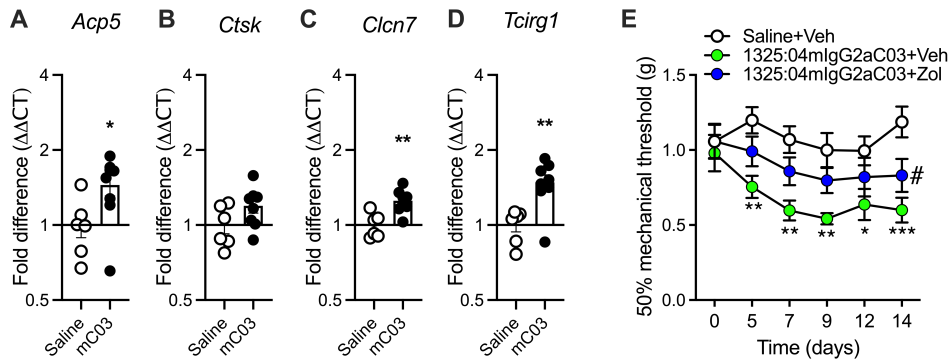

Figure 11. Monoclonal C03-induced mechanical hypersensitivity is associated with increased expression of osteoclast-related genes in the joints and is partially prevented with zoledronate. **(A-D)** Systemic injection of C03 mAb increases the expression of osteoclast-related genes in the ankle joints, compared to control; n=6-8/group. **(E)** Zoledronate (100 ug/kg) administered i.p. on days 1, 4, and 7 post-mAb injection partially prevents the development of C03-induced mechanical hypersensitivity (n=11-16/group). Data presented as mean±SEM, n=6-16/group, *p<0.05, **p<0.01, ***p<0.001. Adapted from **Study II**, Figure 2.

Joint pain is often associated with increased levels of neurotrophic factors in the joint, which can directly potentiate nociceptive signaling and/or stimulate other cells to release proinflammatory factors (398,399). Interestingly, we found that systemic injection of C03 mAb leads to enhanced expression of nerve growth factor (*Ngf*) and neurotrophin 3 (*Ntf3*) in the ankle joints (Study II, Figure 3). Furthermore, neutralizing NGF using anti-NGF monoclonal antibody significantly reversed C03-induced mechanical hypersensitivity in C03-injected mice, compared to mice injected with the vehicle. According to the literature several cells in the joint can produce and release NGF (400–404). Based on previous findings we stimulated human fibroblasts, macrophages, neutrophils and osteoblasts with C03 mAb, but we did not succeed in identifying the source of NGF as none of them released NGF in response to the antibody. Future studies will determine if osteoclasts, chondrocytes or mast cells are the source of NGF in this model.
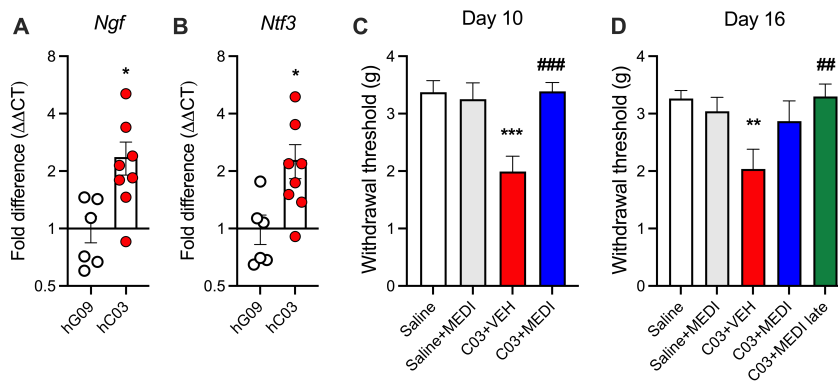
Figure 12. C03-induced mechanical hypersensitivity is associated with increased expression of neurotrophic factors in the joint and can be attenuated using anti-NGF antibody. **(A)** Systemic injection of C03 mAb (2 mg/mouse) increases the expression of *Ngf* and **(B)** *Ntf3* in mouse ankle joints, compared to mice injected with control mAb, n=6-8/group. MEDI-578 (3 mg/kg) was administered s.c. every 6 days, starting from day 3 post-C03 injection until end of the study (shown in blue), or as a single i.p. injection on day 14 post-C03 injection (shown

in green). **(C)** MEDI-578 significantly reversed mechanical hypersensitivity in C03-injected mice assessed on day 10 and **(D)** day 16 post-C03 injection and compared to C03+VEH group; 8-15/group until day 14, n=7-13/group from day 14 until the end of the study. Adapted from **Study II**, Figure 3.

Long-term NGF signaling leads to transcriptional changes in the DRG and enhanced expression of pronociceptive factors. While we have no evidence for direct action of C03 mAb in the DRG, we found that systemic C03 injection upregulates several glia- and inflammation-related genes in L3-L5 DRGs (Study II, Figure 4). These transcriptional changes are most likely a consequence of C03 action in the joint and a local release of NGF that acts on nociceptors in the joint. Finally, we were curious to see if the drugs that attenuated C03-induced pain-like behavior had any effect on the transcriptional changes in the DRGs. Intriguingly, while zoledronate had only partial effect on mechanical hypersensitivity it normalized the expression of almost all studied factors in the DRGs (Study II, Figure 5). In contrast, anti-NGF antibody had no effect on any of the upregulated genes, while reparixin had a mixed effect, with expression of several factors being further enhanced with the treatment.

In conclusion, our work points to a complex interaction between osteoclasts and other cells in the joint that mediates pain-like behavior in response to C03 mAb. Although there are still critical experiments to undertake in order to delineate the exact relationship between osteoclast activation and increased NGF signaling in this model, we speculate that C03 mAb stimulates osteoclasts and leads to the release of CXCL1. This in turn provides signal to surrounding cells to release NGF, which in synergy with CXCL1 activates nociceptors and drives a strong increase in nociceptor signaling. While pain-like behavior induced by polyclonal ACPA is coupled to bone erosion (138), C03 mAb was not sufficient to induce bone loss. Despite that, the role of osteoclasts in inducing mechanical hypersensitivity seems to be critical and is highlighted by the anti-nociceptive effects of zoledronate at both behavioral and molecular level. We find it important that NGF mediates mechanical hypersensitivity even in the absence of inflammation and that increased expression of pro-inflammatory factors occurs at a higher magnitude than in the joint. This observation points to a possibility that NGF and osteoclast-derived ligands contribute to arthralgia in ACPA-positive pre-RA individuals who suffer from joint pain but did not yet develop inflammation.

5.3 STUDY III: ANTI-MODIFIED PROTEIN ANTIBODY INDUCES MECHANICAL HYPERSENSITIVITY VIA FC-GAMMA RECEPTORS

In Study I, we have demonstrated that a combination of two RA monoclonal antibodies (B02/B09) induces mechanical hypersensitivity via an osteoclast-dependent mechanism. Intriguingly, while both mAbs induce mechanical hypersensitivity when injected alone into

mice, B09 mAb does not bind osteoclasts and has no impact on bone metabolism *in vitro* or *in vivo*, which suggests that other mechanisms are behind its pronociceptive properties. Thus, the aim of Study III was to explore the processes through which B09 mAb induces pain-like behavior in mice.

We have replicated our previous findings that systemic injection of B09 mAb induces mechanical hypersensitivity in mice, as well as leads to cold allodynia and causes a drop in withdrawal thresholds for heat stimuli (Study III, Figure 1). Similarly to other RA-autoantibodies, B09 mAb gave rise to pain-like behavior that was not attenuated with NSAIDs like naproxen or diclofenac, but instead was reversed with buprenorphine, a partial opioid agonist. Concomitantly, we did not detect any histological signs of joint inflammation or changes in the expression of pro-inflammatory factors in the ankle joints of B09-injected male or female mice (Study III, Figure II).

To investigate if B09 mAb exerts its pronociceptive action through antigen-binding region (Fab) or by forming immune complex (IC) and activating Fcγ receptors, FcγR$^{-/-}$ and wild-type (WT) mice were injected systemically with B09 mAb. In contrast to WT mice, which developed mechanical hypersensitivity, male and female mice that lacked activating FcγRs were protected from B09-induced mechanical hypersensitivity (Study III, Figure 3). Similar observation was made upon intra-articular injection of B09, which caused a drop in withdrawal thresholds in WT but not FcγR$^{-/-}$ mice. Local action of the antibody could be explained by its binding to antigens present in the ankle joint followed by forming IC and activating FcγRs. Previous studies have demonstrated that B09 mAb binds and enhances the migration of stressed fibroblasts-like synoviocytes through a mechanism that is dependent on antigen binding through Fab fragment (298). Moreover, B09 was shown to form ICs that stimulate peripheral blood mononuclear cells to release pro-inflammatory factors like IL-8 or TNF (390). In our study, mRNA levels for chosen inflammatory cytokines were not increased in the joints, thus this scenario is unlikely, however measuring protein levels of cytokines is needed to make a firm conclusion. We have previously shown that mouse FcγRI is locally translated at the peripheral nerve endings, enabling nociceptors to directly react with ICs present in the vicinity (285). This opens up the possibility of an alternative scenario where B09 forms ICs and induces mechanical hypersensitivity by directly activating FcγRs located on nerve terminals in the joint. Further work is needed to understand the exact interaction between B09 mAb and FcγRs.
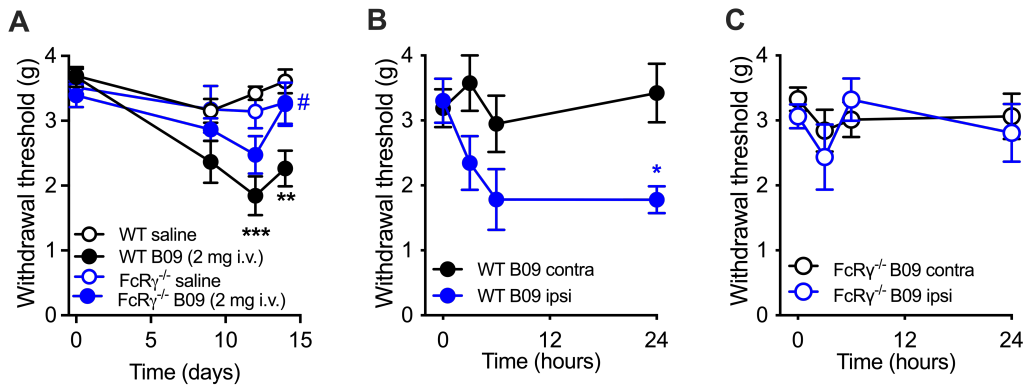
Figure 13. B09-induced mechanical hypersensitivity is dependent on FcγRs. **(A)** FcγR-deficient male mice are protected from mechanical hypersensitivity induced by murine B09 mAb (2 mg/mouse), compared to wild-type mice, n=5-7/group. **(B)** WT male mice develop mechanical hypersensitivity 24 h after i.a. injection of 2.5 μg of murine B09 into the ipsilateral ankle joint, compared to contralateral paw, n=6/group. **(C)** Male mice lacking FcγRs are completely protected from mechanical hypersensitivity induced by administration of B09 mAb into the ipisilateral joint, and compared to contralateral paw, n=6/group. Data presented as mean±SEM, * or # $p<0.05$, **$p<0.01$, ***$p<0.001$. Adapted from **Study III**, Figure 3.

We next asked the question if B09-induced mechanical hypersensitivity is associated with transcriptional changes in the DRGs. We found that systemic B09 injection triggered a pronounced upregulation of several inflammatory factors as well as markers of macrophage, glia and neuronal activation in L3-L5 DRGs (Study III, Figures 4 & 5), but not in the joints or lumbar spinal cord (Study III, Figure S1). Moreover, the lack of FcγRs not only protected the mice from B09-induced pain-like behavior but also normalized the expression of most inflammatory and macrophage-related markers in the DRGs. Of interest, FcγR deficiency did not alter the enhanced expression of satellite glial cell markers and some of the neuronal markers (*e.g. Calca*, *Trpm8*).
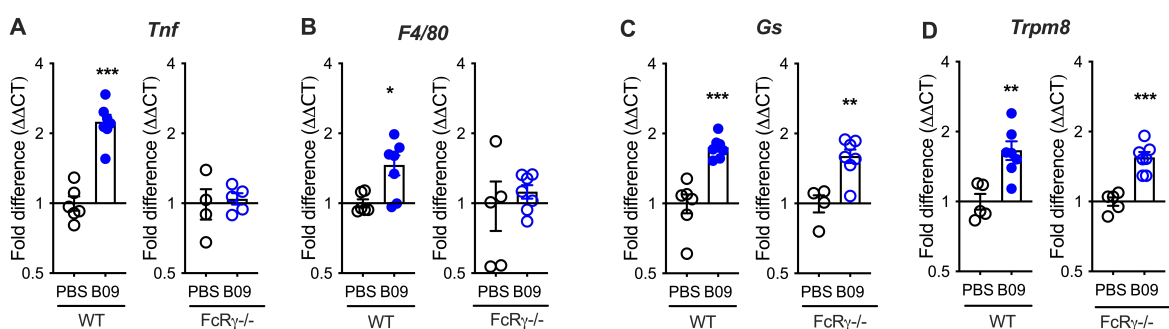
Figure 14. B09-induced upregulation of inflammatory and macrophage related markers, but not SGC or neuronal markers depends on the presence of FcγRs. Examples of **(A)** inflammatory and **(B)** macrophage-related factors significantly upregulated with systemic B09 injection and downregulated in the absence of FcγRs. **(C)** SGC- and **(D)** neuronal markers are upregulated by B09 injection and not affected by the absence of FcγRs. The mRNA data were normalized to *Hprt* mRNA levels and are presented as fold change (n=4-7/group). Data presented as mean±SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control group. Adapted from **Study III**, Figures 4 & 5.

Increasing number of studies points to immune cells like macrophages and glia cells as important modulators of pain behaviors. Based on the findings we made in the DRGs we aimed

to explore if B09 mAb could bind and directly stimulate macrophages and/or satellite glial cells. While we did not see any binding of B09 in primary macrophage cultures and no signs of macrophage infiltration and/or activation in the DRGs upon B09 injection (Study III, Figure 6), we found that B09 mAb binds DRG satellite glial cells *in vitro* (Study III, Figure 7). Of note, since B09 mAb was previously reported to preferentially bind nuclear antigens, we live incubated the cells and observed B09 binding in non-permeabilizing conditions. As a result, we have detected positive binding signal in non-neuronal PGP 9.5-negative and GS positive cells. This is intriguing as SGCs are considered to lack FcγRs, suggesting that B09 mAb binds epitopes on the surface of these cells. Finally, in order to understand if B09 binding exerts any functional effects we have used CXCL1 as a readout of SGC activation and LPS as a "stress trigger". We found that while B09 alone did not stimulate SGCs it acted in synergy with LPS to significantly enhance the release of CXCL1. These results suggest that B09 mAb can stimulate SGCs to release CXCL1 in the presence of certain inflammatory triggers. Further studies are needed to unravel if the same mechanism is present *in vivo*.

To conclude, our findings point to a new, osteoclast-independent mechanism through which a monoclonal RA autoantibody induces pain-like behavior in mice. We find it intriguing that single mAb leads to a global upregulation of several proinflammatory, glia- and neuronal-related factors, which is restricted to DRGs. It suggests, similarly to what we saw with C03 mAb, that inflammatory mediators act locally and are not necessarily accompanied by systemic inflammation. While we have not confirmed that B09 mAb can act locally in the DRG, it is plausible that it binds antigens on SGC surface and activates glia to release pronociceptive factors. Moreover, as FcγRs deficiency normalized proinflammatory- and macrophage-related factors, but not SGC or all neuronal markers we speculate that upon antigen binding, B09 mAb forms ICs that will concomitantly stimulate resident macrophages in the DRG. The scenario in which SGCs are directly stimulated by B09 mAb could explain why in the absence of FcγRs we still observe transcriptional signs of SGC and neuronal activation.

5.4 STUDY IV: ANTIBODIES FROM FIBROMYALGIA PATIENTS MEDIATE HYPERSENSITIVITY IN MICE

Fibromyalgia syndrome (FMS) is a chronic condition characterized by widespread pain, tenderness, fatigue and emotional disturbances. While the exact etiology and pathophysiology are unknown, it has been suggested that FMS might be associated with immune dysregulation. Alterations in pro- and anti-inflammatory cytokine found in FMS patients, as well as high comorbidity of FMS in autoimmune diseases led us to postulate that FMS may be driven by autoimmune mechanisms. Thus, the aim of Study IV was to investigate if IgG antibodies purified from FMS patients mediate FMS symptoms when administered to mice.
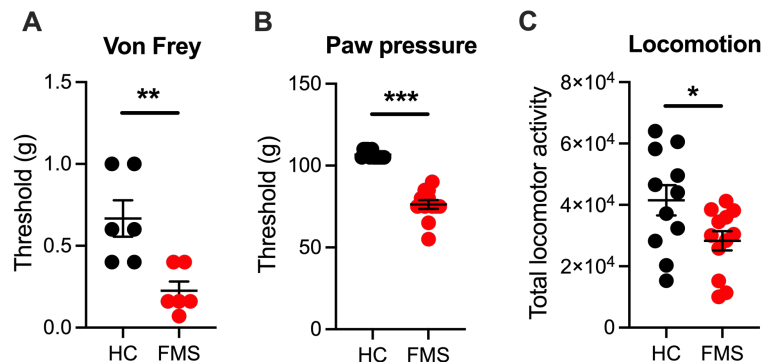
Figure 15. Passive transfer of FMS IgG induces hypersensitivity and reduces locomotion in mice. Mice injected with FM IgG were hypersensitive to **(A)** punctate stimuli (Von Frey) and **(B)** paw pressure (Randall-Selitto) four days after injection, n=6/group. **(C)** FMS IgG-injected mice were less active during the peak activity phase (22:00-2:00) five days after injection, compared to HC IgG injected mice, n=12/group. Data presented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001 compared with control group. Adapted from **Study IV**.

Firstly, IgG purified from the serum of FMS patients and healthy controls (HC) was administered to female mice through i.p. injection on 4 consecutive days (8 mg per day). Since FMS patients often exhibit polymodal hypersensitivities, we have tested the mice for different sensory modalities as well as measured the evoked and non-evoked pain behaviors. We found that injection of FMS IgG, but not HC IgG led to decreased mechanical pressure thresholds in hind paws and thighs of the mice as well as increased sensitivity to noxious cold, which developed within 24-48 hours after first IgG injection and lasted for approximately a week after the final injection (Study IV, Figure 1, 2). Furthermore, mice injected with FMS IgG exhibited decreased mechanical thresholds to stimulation with von Frey filaments, reduced grip strength as well as decreased activity at night (time when mice are usually most active), compared to mice injected with HC IgG. These results demonstrate that IgG mediates the key features of FMS: pressure and cold sensitivity, muscle weakness and fatigue, reflected by the impact on both evoked and non-evoked mice behaviors. Importantly, these findings were reproduced using IgG from individual patients as well as using IgG preparations pooled from FMS patients from a different and regionally distinct cohort. Of note, IgG-depleted serum of FMS patients did not produce cold or mechanical hypersensitivity, pointing to the functional role of IgG in

mediating FMS symptoms (Study IV, Figure 4). Furthermore, male and female mice exhibited comparable mechanical and cold hypersensitivity after FMS IgG injection, indicating that a higher prevalence of FMS among women is most probably not due to sexual dimorphism or increased sensitivity to IgG in females.

Sensory abnormalities in FMS patients might be partially explained by the sensitization of C-fibers, detected using microneurography (345). Intriguingly we found that Aδ- and C-mechanosensitive fibers in skin-nerve preparations from mice injected with FMS IgG had reduced mechanical response thresholds compared to preparations from HC IgG-treated mice (Study IV, Figure 6). While pain in some autoimmune diseases is caused by a direct activation of sensory neurons by IgGs (16), using calcium imaging, we have excluded the possibility that FMS IgG directly acts on sensory neurons in the DRGs and leads to hypersensitivity. Instead, immunohistochemical and Western Blot analysis demonstrated a robust accumulation of FMS IgG in the lumbar DRGs, but none in the spinal cord or brain, with FMS IgG binding having a higher pixel intensity and staining larger areas compared to HC IgG (Study IV, Figure 7). As shown with anti-glutamine synthetase (GS) staining, FMS IgG primarily colocalized with SGCs surrounding the sensory neurons in the DRGs, however it was also detectable in fiber tracts and a small number of Iba-1 positive macrophages and CD31 positive blood vessels. Although we did not detect a direct effect of FMS IgG on sensory neurons, IHC has shown extensive labelling of GS-positive cells. In addition, FMS IgG binds some, but not all, TrkA-positive neurons in the DRGs. While IHC limits the possibilities of differentiating between labelling SGCs and sensory neurons we have performed a series of *in vitro* ICC experiments using primary DRG neuron-rich and SGC-enriched cultures. We have demonstrated that FMS IgG binds a higher proportion of cells in both DRG neuron-rich and SGC-enriched cultures, compared to HC IgG without affecting their viability. Moreover, by live-staining the cells with IB4, a membrane marker of a certain nociceptor subpopulation, we confirmed that FMS IgG binds neuronal membranes. Although we cannot directly translate *in vitro* findings to *in vivo* processes, this experiment supports the scenario where FMS IgG binds both SGCs and sensory neurons in the DRG.

We next investigated if FMS IgG binding to SGCs *in vivo* has any functional consequences. We have detected increased signs of SGC activation – enhanced GFAP staining as well as increased *Gfap* and *S100β* expression in the DRGS from mice injected with FMS IgG, compared to HC IgG controls. Interestingly, while we did not detect increased Iba-1 staining in response to FMS IgG accumulation, we still found *Itgam* and *Aif1* (macrophage markers) mRNA to be increased in the DRGs from FMS IgG-injected mice (Study IV, Figure 8). This

suggests that although there is no evident macrophage infiltration and/or proliferation in the DRG, the presence of FMS IgG might still induce molecular changes in the surrounding cells.

While several human studies reported altered levels of cytokines in the serum or plasma of FMS patients, we did not find any differences in the levels of TNF-α, IL-2, IL-5, IL-6, IL-10, IFN-γ or CXCL1 in the serum from FMS IgG, HC IgG, or saline-injected mice (Study IV, Figure 8). On the other hand, FMS patients were reported to have signs of small-nerve fiber pathology as well as reduced skin innervation, which might contribute to their hypersensitivity (344). In line with human data, we found that FMS IgG led to a decrease in intra-epidermal nerve fiber density (IENFD) in the glabrous skin of the mouse hind paw (Study IV, Figure 10), suggesting a possible role of IgG in reduced innervation. Finally, we have stained human DRG sections with FMS IgG and HC IgG and found that FMS IgG bound GFAP- and NF200- immunoreactive cells, demonstrating that autoreactive FMS IgG binds antigens expressed on human SGCs and sensory neurons.
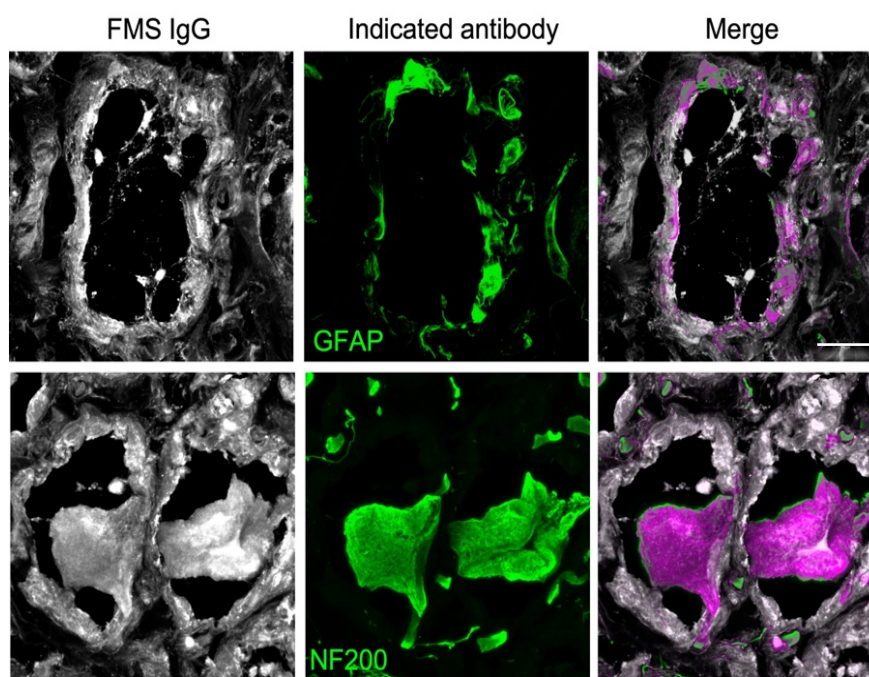
Figure 16. FMS IgG binds human DRG. High-magnification images of FMS IgG (white) show colocalization (purple) between FMS IgG and GFAP-positive SGCs (green) and NF-200-immunoreactive neurons (green). Scale bar 20 μm. Adapted from **Study IV**, Figure 11.

To summarize, we have shown for the first time that passive transfer of fibromyalgia IgG can mediate key features of the disease in mice. While IgG from HC and/or IgG-depleted serum did not evoke any pain-like behaviors in mice, we postulate that the multimodal sensitivity and tenderness seen in FM patients, depends on the presence of autoreactive IgGs. In addition to increased sensitivity, FMS IgG transfer led to muscle weakness, diminished activity and decreased skin innervation, pathophysiological features often seen in FMS patients. Albeit FMS is thought to be mainly driven by alterations in the CNS, our work points to possible

contribution of peripheral disturbances demonstrated by sensitization of peripheral nociceptors and reduced skin innervation. Moreover, enhanced responsiveness of nociceptors located in the skin collected from mice injected with FMS IgG, shows that increased neuronal firing induced by the antibodies persists even without the connection to the CNS. Intriguingly, while consequences of FMS IgG administration are global, DRGs seem to be the only site of action for the autoreactive IgG. We speculate that upon binding to specific epitopes on SGCs, FMS IgG activates SGCs that can further release pronociceptive cytokines and enhance nociceptor excitability resulting in hypersensitivity. Moreover, the presence of autoreactive IgGs in the DRG will indirectly stimulate resident macrophages and further contribute to neuronal overexcitation. Importantly, our findings open the possibilities for treatments of FMS that were not previously considered. Although more studies are needed to uncover the specific targets of the FMS IgG which will allow for specific removal of autoreactive antibodies or blockage of their action, therapies that decrease amount of circulating IgG might prove efficacious already now.

6 CONCLUDING REMARKS

Pain in musculoskeletal diseases like RA or FMS continues to be a huge problem that is poorly managed with available therapies. Recent years have brought evidence for a bidirectional communication between the sensory system and the immune system in initiating and sustaining chronic pain. For example, it has been shown that autoantibodies actively mediate persistent pain in several chronic conditions through their interaction with immune receptors, complement system or by binding epitopes or FcγRs on sensory neurons. The focus of this thesis was to further delineate the mechanisms through which autoantibodies isolated from serum of RA and FMS patients induce pain behaviors in mice, with the hope to identify novel targets or strategies that could serve as a basis for development of new pain therapeutics.

Study I and II point to an important role of osteoclast activation in driving pain in RA and a possible use of osteoclast inhibitors as novel treatment of arthralgia. In **Study I** we have used a combination of two mAbs RA autoantibodies with distinct functional properties – one with mild osteoclastogenic properties and the other with fibroblast-binding capabilities. Intriguingly, only when combined these mAbs induced prolonged mechanical hypersensitivity and potently activated osteoclasts leading to bone erosion. This suggests that the synergistic mechanisms of action of these two mAbs are needed to potently stimulate bone metabolism in this model. This observation is particularly important as RA patients often harbor a wide spectrum of autoantibodies. Pin-pointing the exact interactions between them could open up for novel avenues to treat bone erosion and pain in RA.

While we did not find evidence for proton-driven pain-like behavior in **Study I** we have identified LPC 16:0 and ASIC3 as mediators of autoantibody pain-like behavior in this model. Intriguingly, high levels of LPC 16:0, as well as an overactivation of the conversion pathway from phosphatidyl choline to LPC have been previously reported in patients with painful joint diseases, including RA (48,391,405), suggesting that LPC might be a relevant target for pain-relief. However, the cellular sources of sPLA$_2$ and LPC and the exact mechanisms that trigger sPLA$_2$ activation and subsequent LPC production in the joint remain to be discovered. Of note, LPC 16:0-induced ASIC3 sensitization was previously reported as one of the mechanisms driving pain-like behavior in animal models of fibromyalgia (406–408). Intriguingly, clinical metabolomic analyses showed that FMS patients have increased levels of lysophosphatidylcholine metabolites (including LPC 16:0) in their plasma, compared to controls (409,410). Although we have not assessed the levels of LPC in the plasma of FMS IgG-injected mice in **Study IV**, it would be worthwhile to investigate if FMS IgG leads to

enhanced production of LPC 16:0 that could sensitize ASIC3 and contribute to hypersensitivity in this model. While authors of the previously mentioned studies suggest that high degree of oxidative stress and peroxidation of lipids might be specific for FMS pathogenesis, further studies are warranted to understand if LPC 16:0–induced ASIC3 sensitization is a common mechanism in chronic pain conditions like RA and FMS or if it is associated with the activation of disease-specific pain pathways.

In **Study II** we have identified a link between autoantibody-induced altered bone metabolism and NGF-driven nociception. While we did not detect bone erosion *in vivo*, blocking osteoclast activity partially reversed pain-like behavior and prevented the transcriptional changes in the DRGs, suggesting a close relationship between enhanced bone metabolism and NGF signaling. Indeed, NGF was previously shown to stimulate osteoclastogenesis even in the absence of RANKL (411), suggesting that increased levels of NGF can concomitantly contribute to C03-induced osteoclast activation. The cellular source of NGF in this model was not deciphered in **Study II** and remains to be found. Understanding which cell type in the joint contributes to NGF/C03-induced hypersensitivity will allow for a more precise targeting of this pronociceptive mechanism. Our findings have translational value as they are in line with human studies showing increased levels of NGF in synovial fluid samples from painful joint diseases like OA, RA or JIA (412–414). Several approaches have been developed to target NGF signaling, including sequestration of free NGF, prevention of its binding to TrkA receptor or inhibition of TrkA function (415). Many of these compounds have reached late-stage development and have showed a significant analgesic effect over placebo in clinical trials (416,417). However, anti-NGF therapies have been associated with high risks of joint destruction which halted their entry into the market. This observation further points to an involvement of NGF signaling in bone remodeling and underlines the urgency of identifying the exact role of NGF in bone physiology and pathology for the success of future anti-NGF analgesic therapies.

Study III demonstrates an important role of FcγRs in mediating autoantibody pain-like behavior. Earlier work from our laboratory and other research groups has shown that autoantibodies in IC formation bind to neuronally expressed FcγRs and contribute to mechanical hypersensitivity (278,285,418). Although we have not yet identified if this is the exact mechanism behind B09 mAb-induced pain-like behavior, its direct effect in the joint and complete protection from pain-like behavior in FcγRs-deficient mice supports this notion. Thus, future studies are required to understand if B09 mAb forms ICs in the joint and activates FcγRI on sensory neurons or if other FcγR-dependent mechanisms are at play. Most of the

currently used RA therapies do not directly alter FcγRs function, but instead dampen the response of the immune system. Based on our findings we propose that specific targeting of neuronally expressed FcγRs could offer a novel approach for the treatment of autoantibody-induced pain which is not accompanied by inflammation.
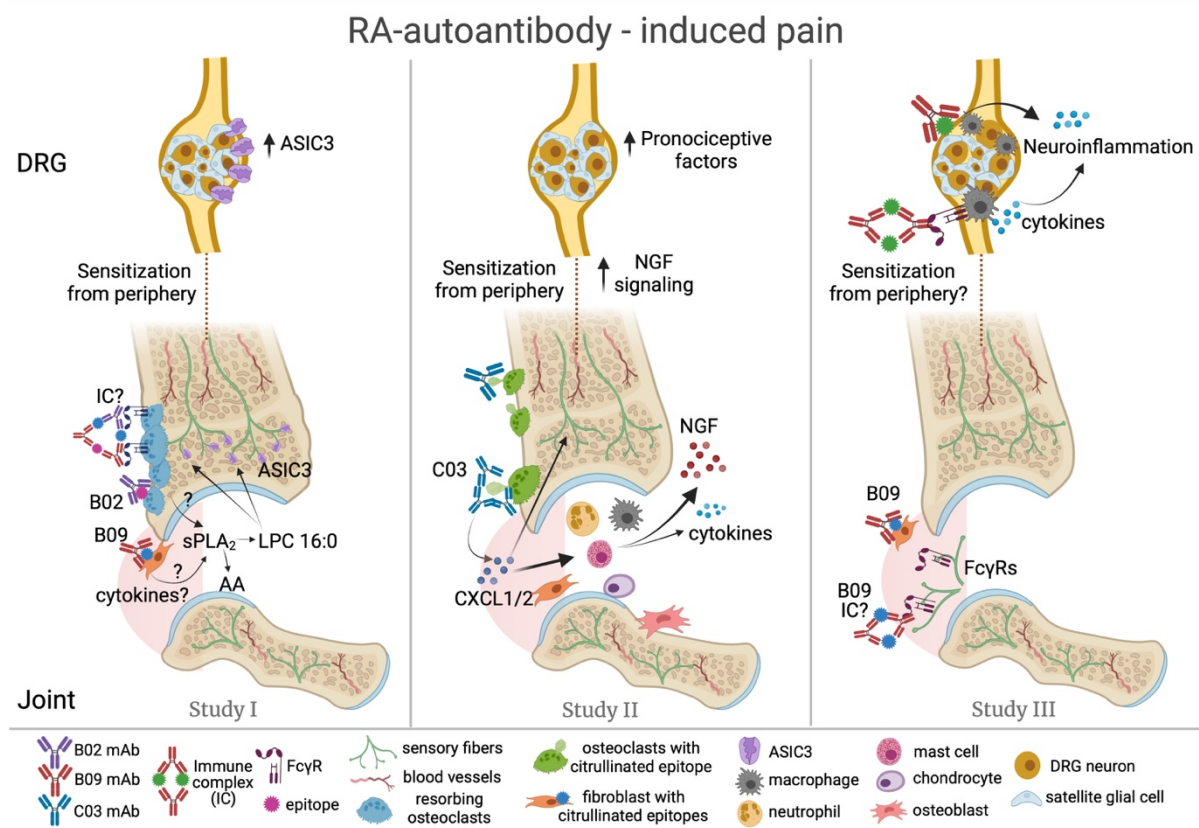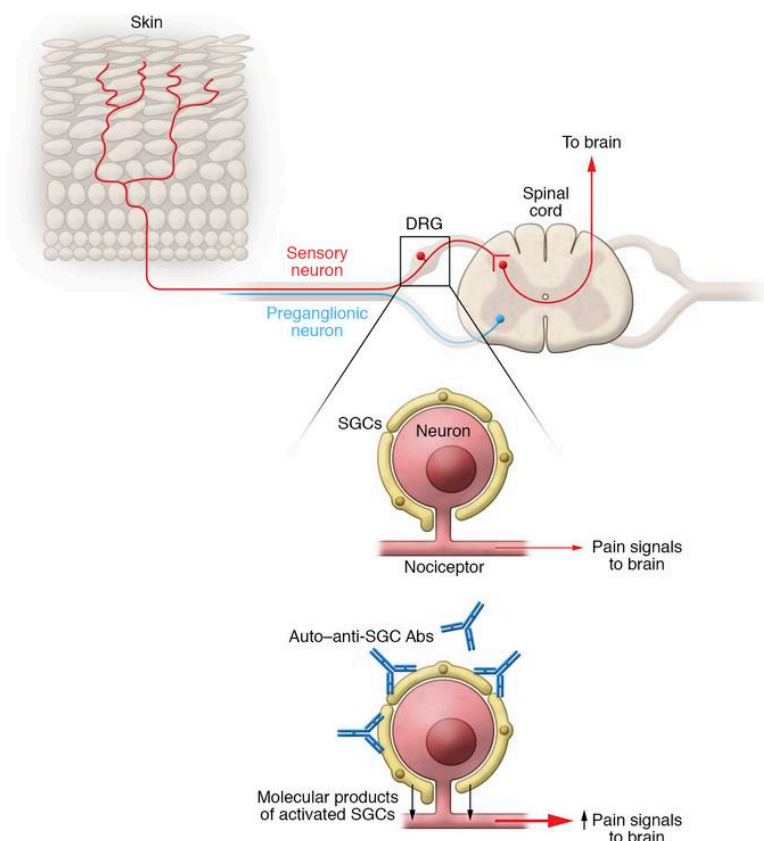

Figure 17. RA-associated autoantibodies induce mechanical hypersensitivity through various mechanisms. In **Study I** we have demonstrated that B02 and B09 mAbs stimulate osteoclasts and lead to the development of pain-like behavior and bone erosion. Mechanical hypersensitivity in this model is associated with increased ASIC3 expression in the DRGs and can be reversed by blocking ASIC3 signaling. Increased sPLA2 activity and enhanced levels of LPC 16:0 contribute to sensitization in this model. In **Study II** we have linked C03-induced mechanical hypersensitivity to increased NGF signaling. We speculate that CXCL1/2 released from activated osteoclasts acts in synergy with NGF and contributes to nociceptor sensitization. Enhanced nociceptor signaling in the joint leads to transcriptional changes in the L3-L5 DRGs and to pain-like behavior in this model. **Study III** points to important role of FcγRs in mediating autoantibody-induced pain-like behavior. We speculate that B09 mAb acts on FcγRs in the joint leading to mechanical hypersensitivity. B09 mAb might also bind epitopes on SGC *in vivo* and stimulate them to release pronociceptive factors. Concomitantly, in case of IC formation, B09 mAb can stimulate resident macrophages in the DRG through FcγRI located on their surface, to release proinflammatory factors that further sensitize sensory neurons. Created with Biorender.com.

A common and noteworthy finding made in **Studies I, II and III** is that pain-like behavior induced by RA-associated autoantibodies is not reversible with NSAIDs, commonly used to treat inflammatory pain in the clinic (5,214). While the autoantibodies used in these three studies do not employ the same molecular mechanisms, pain resulting from their action does not depend on "classical" inflammatory processes. Our data using osteoclast-activating RA-autoantibodies points to osteoclast-blocking therapies as efficient alternatives for treatment of

non-inflammatory pain arising from enhanced bone metabolism. However, findings from **Study III** show that RA-associated autoantibodies can also exert their pronociceptive effects through alternative, osteoclast-independent mechanisms. This highlights the notion that autoantibody-induced pain mechanisms in RA are complex and enhanced bone metabolism should not be the only target for pain relief. Extensive studies are warranted to delineate alternative mechanisms through which RA-associated autoantibodies induce pain. This knowledge will help in designing novel, better treatments of pain that does not respond to conventional analgesics.

Figure 18. Cartoon summarizing findings from **Study IV**. Autoreactive IgG antibodies bind SGCs surrounding sensory neurons in the DRGs and subsequentially stimulate sensory neurons to further amplify pain signals. Reprinted with permission and adapted from JCI, Tracey K., July 2021.

Study IV proposes that autoreactive IgG mediates hypersensitivity in fibromyalgia. We have demonstrated that passive transfer of FMS IgG to mice leads to multimodal hypersensitivity, motor symptoms and decreased skin innervation. Extensive preclinical research is still needed to understand how FMS IgG drives hypersensitivity and if its mechanism of action is solely based on epitope binding on SGCs or if FcγR-dependent mechanisms are involved. Furthermore, understanding if FMS IgG-induced peripheral sensitization can cause alterations in the CNS (*e.g.* enhanced glial activation and/or altered signaling) which are seen in FMS patients will further increase the translational value of these findings. Discoveries made in **Study IV** might have a profound effect on future treatment options for patients with fibromyalgia. While historically fibromyalgia has been viewed as "pain in the brain" and no therapies have been able to offer an effective management of pain symptoms, our findings open the possibility of using immunomodulatory therapies. A single clinical study provided promising data showing that IVIG therapy decreased pain and tenderness as well as increased muscle strength in FMS

patients (374). Continuing preclinical research on autoimmune mechanisms behind fibromyalgia will allow for better, mechanism-based treatments in the future.

In conclusion, a shared characteristic of all four studies described in this thesis is their translational approach to studying pain mechanisms. We have shown that passive transfer of autoantibodies into mice allows for partial transfer of disease-related symptoms, which opens the possibility of identifying responsible processes for pain induction and maintenance in diseases like RA or FMS. Using the bedside-to-bench approach in all four studies, we have provided several interesting cellular and molecular insights into the possible mechanisms behind autoantibody-induced pain. While more studies are needed to confirm the translational value of our findings, the work described in this thesis brings us one step forward in creating more effective pain therapies for several chronic diseases.

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This thesis represents not only the science behind my PhD but, more importantly, it reflects the life journey I undertook in 2015 by coming to Sweden and to Karolinska Institutet. I wouldn't be where I am now – both scientifically and on a personal level – if it was not for the wonderful and valuable people I met during these years, who on different steps of my way helped me either with experiments or through exchanging scientific ideas, having inspirational conversations, or simply sharing some goofy laughs over lunch. All these things – big and small, helped me survive the tough periods of the PhD as well as enjoy the good ones, and I am forever grateful for being surrounded by all of you. Thank you!

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