MECHANISMS OF PAIN IN AUTOIMMUNITY – THE ROLE OF ANTIBODIES

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Mechanisms of pain in autoimmunity – the role of antibodies

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ABSTRACT

Chronic pain in autoimmune diseases, like rheumatoid arthritis (RA), is a common and life-changing problem for many patients. Treatment is usually aimed at reducing inflammation and preserving the function of affected tissues. Chronic pain, however, often persists despite optimal disease control. Autoimmune pain arises from multiple mechanisms with a wide range of characteristics that differs between individuals. For effective management of the pain, it is essential to understand these mechanisms.

One of the hallmarks in the pathogenesis in most autoimmune diseases is the presence of autoantibodies. In RA, several types of antibodies are well characterized, but little is known about their interaction with the sensory system. Thus, the aim of this thesis is to explore mechanisms involved in pain signaling, specifically the role of disease-relevant antibodies as inducers of pain.

In Paper I and II, we investigate the effect of anti-citrullinated protein antibodies (ACPA) on pain behavior and interaction with immune cells. When injected into mice, both polyclonal human ACPA or murinized monoclonal ACPA induces spontaneous and evoked pain-like behavior in the absence of inflammation. Additionally, the antibodies induce trabecular bone loss measured with micro-CT. The antibodies localize to joint and bone marrow, binding osteoclasts and its precursors. Using cultures of mice and human osteoclasts, we show that ACPA bind structures on the cells, causing proliferation and release of the chemokine CXCL1/IL-8. The effect of the release is increased bone resorption and activation of sensory neurons, causing pain-like behavior, which can be reversed by treating the mice with the CXCR1/2 blocker reparixin.

In Paper III, we demonstrate that mice injected with antibodies specific to the cartilage protein collagen type II (anti-CII mAbs) displays pronounced mechanical hypersensitivity and reduction in locomotion at time points when visual, histological and molecular indications of inflammation were completely absent. Further, this effect was not mediated by the activation of complement factors or by changes in the cartilage structure. Instead, our data point to a direct action of anti-CII mAb/collagen immune complexes on the sensory neurons through neuronally expressed Fc-gamma receptor IIb (FcγRIIb), causing increased inward currents, intracellular Ca\(^{2+}\) levels, and calcitonin-gene related peptide (CGRP) release. Importantly, the nociceptive properties of anti-CII mAbs were lost when the Fc-FcγR interaction was disrupted in vivo.

In summary, we have described two novel mechanisms of how disease-relevant antibodies can activate sensory neurons, causing pain-like behavior. These results deepen the understanding of pain mechanisms in autoimmune disease and potentially to new ways of treating the pain component in patients.
LIST OF SCIENTIFIC PAPERS

Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. 
*Annals of the Rheumatic Diseases* (2016) –208094


Collagen type II specific antibodies induce pain through immune complex mediated stimulation of neurons
Manuscript
Publications not included in the thesis

I. Codeluppi S, Gregory EN, Kjell J, **Wigerblad G**, Olson L, Svensson CI.
   **Influence of rat substrain and growth conditions on the characteristics of primary cultures of adult rat spinal cord astrocytes.**
   *Journal of Neuroscience Methods*, (2011) 197(1), 127

II. Abdelmoaty S, **Wigerblad G**, Bas DB, Codeluppi S, Fernandez-Zafra T, El-Awady ES, Moustafa Y, Abdelhamid AEDS, Brodin E, Svensson CI.
   **Spinal Actions of Lipoxin A4 and 17(R)-Resolvin D1 Attenuate Inflammation-Induced Mechanical Hypersensitivity and Spinal TNF Release.**
   *PloS One*, (2013) 8(9), e75543

III. Norsted Gregory E, Delaney A, Abdelmoaty S, Bas DB, Codeluppi S, **Wigerblad G**, Svensson CI.
   **Pentoxifylline and propentofylline prevent proliferation and activation of the mammalian target of rapamycin and mitogen activated protein kinase in cultured spinal astrocytes.**
   *Journal of Neuroscience Research*, (2013) 91(2), 300–312

   **Olfactory exposure to males, including men, causes stress and related analgesia in rodents.**

V. Bas DB, Su J, **Wigerblad G**, Svensson CI.
   **Pain in rheumatoid arthritis: models and mechanisms.**
   *Pain Management*, (2016) 6(3), 265–284

   **Inflammation-induced GluA1 trafficking and membrane insertion of Ca\(^{2+}\) permeable AMPA receptors in dorsal horn neurons is dependent on spinal tumor necrosis factor, PI3 kinase and protein kinase A.**
   *Manuscript/submitted*
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<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACPA</td>
<td>Anti citrullinated protein antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American College for Rheumatology</td>
</tr>
<tr>
<td>AIA</td>
<td>Adjuvant-induced arthritis</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>CII</td>
<td>Collagen type II</td>
</tr>
<tr>
<td>CIX</td>
<td>Collagen type IX</td>
</tr>
<tr>
<td>CAIA</td>
<td>Collagen antibody-induced arthritis</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>Cit</td>
<td>Citrulline/Citrullinated</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine CXC motif ligand</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease modifying anti-rheumatic drug</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FT</td>
<td>Flow through</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy controls</td>
</tr>
<tr>
<td>IB4</td>
<td>Isolectin B4</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine based inhibitory motif</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactive</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>K/BxN</td>
<td>K/BxN serum transfer model</td>
</tr>
<tr>
<td>MCP</td>
<td>Metacarpophalangeal</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTP</td>
<td>Metatarsophalangeal</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal interphalangeal</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>YLD</td>
<td>Years lived with disability</td>
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1 INTRODUCTION

Chronic pain is a major health problem affecting a large portion of the population, causing a marked reduction in the quality of life for the individual as well as large socioeconomic costs. It has been estimated that chronic pain conditions are the major contributor to Years lived with disability (YLD) globally (Vos et al., 2012), where pain due to muscoskeletal joint disorders like osteoarthritis (OA) and rheumatoid arthritis (RA) are common causes (Breivik et al., 2006; Vos et al., 2012). RA is a systemic autoimmune disease that affects around 1% of the population. Recent enhanced understanding of the molecular pathogenesis has greatly improved treatment outcomes (Firestein, 2003; McInnes and Schett, 2007), but many patients still suffer from joint pain (Altawil et al., 2016; Lee et al., 2011). Pharmacological treatments of pain are often associated with side effects and lack of efficacy, making pain management clinically challenging (Walsh and McWilliams, 2014). Therefore it is critical to increase our understanding of the mechanisms establishing chronic pain so that we can identify novel targets and treatment strategies for more effective pain relief.

1.1 RHEUMATOID ARTHRITIS

RA is a systemic autoimmune disease where the autoreactivity primarily affects the joints, causing pain and swelling. Pain and stiffness is often increased in the morning and after periods of rest. The course of the disease varies greatly, some patients have mild short-term symptoms but most have progressive life-long symptoms. RA is present in all human populations with some regional differences, with the highest prevalence in Western Europe, affecting women approximately three times more than men (Cross et al., 2014; Firestein, 2003; Klareskog et al., 2009; Scott et al., 2010).

1.1.1 Criteria

Arthritis means inflammation in the joints and is a symptom of many different diseases, including osteoarthritis, psoriatic arthritis, and gout. In order to study RA in a meaningful way it is important to cluster patients and to have uniform criteria for the definition of the disease. The most recent classification criteria for RA were delineated in 2010 by ACR and EULAR (Aletaha et al., 2010) (see Table 1). Patients that are scored need to satisfy two criteria: 1) have at least 1 joint with definite clinical synovitis that 2) cannot be better explained by another disease. A score of 6 out of maximum 10 is defined as having RA. Points are given for number of joints involved and presence of autoantibodies, anti-citrullinated protein antibodies (ACPA) or rheumatoid factor (RF) are given more weight in the scoring. The term seropositive refers to presence of either ACPA or RF, this does not exclude seronegative patients from having other types of autoantibodies. Importantly, in the definition for “involvement”, tenderness or pain is included as an equal important feature as swelling, indicating that the sensory component is an integral part of the disease process. It is important to note that RA is a very heterogeneous disease or syndrome with several patient...
subsets that present with similar symptoms, i.e. inflammation in the joints. This is true also in animal studies where arthritis-like symptoms can be triggered in a number of ways. Different molecular pathways and processes are likely to be activated in different patients (Klareskog et al., 2013b), contributing to the heterogeneity.

Table 1 The 2010 ACR/EULAR classification criteria for RA

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Manifestations</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Joint involvement</td>
<td>1 large joint</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2-4 large joints</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1-3 small joints</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4-10 small joints</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt;10 joints (at least one small joint)</td>
<td>5</td>
</tr>
<tr>
<td>B. Serology</td>
<td>Negative RF and negative ACPA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Low-positive RF or low-positive ACPA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>High-positive RF or high-positive ACPA</td>
<td>3</td>
</tr>
<tr>
<td>C. Acute-phase reactants</td>
<td>Normal CRP and normal ESR</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Abnormal CRP or abnormal ESR</td>
<td>1</td>
</tr>
<tr>
<td>D. Duration of symptoms</td>
<td>&lt;6 weeks</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≥6 weeks</td>
<td>1</td>
</tr>
</tbody>
</table>

Total score of at least 6 out of 10 is defined as RA. Involved joint means swelling or tenderness. Large joint refers to shoulders, elbows, hips, knees, and ankles. Small joint refers to MCP, PIP, second to fifth MTP, thumb interphalangeal joints and the wrist.

1.1.2 Etiology

The cause of RA is not clear but several risk factors have been identified. It is a complex genetic disease, meaning that it involves several genes, environmental triggers and chance. In twin studies, the genetic risk have been estimated to 50% (MacGregor et al., 2000). The genes that are strongly associated in disease are often involved in the function of the immune system, especially T cell activation and the NF-κB pathway (McInnes and Schett, 2011). Seropositive RA is especially associated with a conserved amino acid motif in the HLA-DRB1 region called shared epitope (SE) (Gregersen et al., 1987), suggesting that predisposing T cell selection, antigen presentation or peptide affinity has a role in formation.
of pathogenic autoantibodies. It is still not known where or how the disease is initiated but important information can come from environmental risk factors. Several factors have been implicated, like periodontitis (Mercado et al., 2000) and particular strains of gut microbiota (Scher et al., 2013), but the strongest evidence is linked to the lungs, involving cigarette smoking (Symmons et al., 1997) and other airway exposures (Klockars et al., 1987). Recently a strong risk interaction was found between HLA-DR and smoking in seropositive patients, linking genes and environment (Klareskog et al., 2006). This suggests that seropositive patients have a fundamentally different disease from seronegative patients.

### 1.1.3 Disease course

#### 1.1.3.1 “Pre-RA”

Rheumatoid arthritis is a disease that develops over time, with several events taking place before the onset of symptoms and subsequent diagnosis. This phase is often referred to as “pre-articular” or “pre-RA”. Studies have shown the presence of several types of autoantibodies up to 10 years before the onset of disease (Kurki et al., 1992; Nielen et al., 2004; Rantapää-Dahlqvist et al., 2003), with increasing titers and epitope spreading over time. Interestingly, this phase is associated with little to no detectable inflammation but with arthralgia as an early symptom (de Hair et al., 2014; van Baarsen et al., 2013). This means that the initial formation of autoimmunity is a very early event that most likely is triggered outside the joint (Klareskog et al., 2013a), that can transition to arthralgia and autoimmune disease involving the joints. Little is know of the molecular mechanisms behind these transitions since most genetic and environmental studies are performed on established RA populations (Catrina et al., 2016; 2014; Klareskog et al., 2013a). Increased understanding of the events leading up to diagnosis would provide opportunities for interventions that could efficiently reduce symptoms and potentially inhibit progression.

#### 1.1.3.2 Established RA

In RA the pathogenic autoreactivity primarily targets structures in the joints and is characterized by the presence of several types of autoantibodies that are used both for diagnosis and sub-categorization of patients. The autoantibodies bind epitopes in the synovia causing recruitment and activation of lymphocytes, monocytes and fibroblasts. In early rheumatoid arthritis, the small joints in hands and feet are predominantly targeted but as the disease progresses also larger joints such as the wrists, knees, ankles and hips are also affected, leading to pain, inflammation and hyperplasia of the synovia, cartilage and bone destruction (Fig. 1). The presence of pannus, an aggressive invasive front of various cells, eventually invades and destroys local surrounding tissues, causing loss of joint function (Firestein, 2003; McInnes and Schett, 2011). About 40% of patients present with extra-articular systemic effects like cardiovascular illness, pulmonary disorders, and reduced cognitive function, likely due circulating cytokines and immune complexes (ICs) (McInnes and Schett, 2011). Established RA is often polycyclic, with fluctuating disease
activity over time, but can also be monocyclic, with one episode that ends within a couple of years after diagnosis. Other patients have a progressive RA that continues to increase in severity over time (Graudal et al., 1998; Masi et al., 1976; Pincus and Callahan, 1993).

Figure 1. The normal joint and changes in RA. The synovial joint is composed of two adjacent bone ends in apposition, each covered with a layer of cartilage separated by a joint space that contains the synovial fluid (a). The arthritic joint is characterized by synovitis, influx and local activation of a variety of immune cells. The synovia becomes hyperplastic, cartilage becomes eroded, and pannus infiltrates the bone. Destruction often starts at the cartilage-bone-synovial membrane junction (b). Modified from (Smolen and Steiner, 2003).

1.1.4 Antibodies in RA

Autoimmune diseases are associated with loss of immunological tolerance, which is the ability of the immune system to separate “self” from “non-self”. Lymphocytes are made to react with an enormous variety of antigens, some of which will be self-structures. Historically, the general idea was that all autoreactive B and T cells that reacted with self-structures were deleted leaving only cells that were specific for foreign antigens. The present view is that a low level of autoreactivity is necessary for normal immune function (Dighiero and Rose, 1999), crucial for maintaining populations of lymphocytes in the periphery. It is speculated that lymphocytes have evolved to respond to antigens only in the presence of certain microenvironments, containing inflammatory cytokines (Silverstein and Rose, 2000). This means that development of autoimmune disease is not only dependent on dysfunction of immunological tolerance, but require several events after the formation of autoreactive immune cells.

High titers of circulating autoantibodies is common in many autoimmune diseases and is often used as a diagnostic criteria (see Table 1) but their direct involvement in pathogenesis is not always clear. However, in RA the importance of antibodies and B cells in pathogenesis is supported not only from the original finding of rheumatoid factors (RF) (Franklin et al., 1957), and its pathogenic potential (Sutton et al., 2000), but also from the observation that arthritis is mediated in experimental animals via B cells and by passive transfer of antibodies
Patients display a wide variety of antibody specificities and the antibody profile may sometimes predict disease progression and treatment response, for example seropositive RA has a worse prognosis than seronegative RA (van der Helm-van Mil et al., 2005). Researchers further improve the existing predictive models by integrating the antibody specificities with the known genetic and environmental risk factors.

The model of RA as an autoimmune disease caused by antibodies dictates that antibodies bind epitopes in and around the joint causing formation of immune complexes (ICs) that drive the disease through activation of resident cells (macrophages, FLS) and complement fixation. This leads to subsequent release of chemotactic factors, which recruit immune cells (neutrophils, monocytes) to the joint. Both resident cells and infiltrating cells are activated by the ICs through Fc receptors (FcRs), which perpetuate the inflammatory process leading to tissue destruction (Firestein, 2003; Nandakumar and Holmdahl, 2006). Knowledge about the direct pathogenic potential of individual antibodies comes from animal studies and will be discussed more in section 1.3.

### 1.1.4.1 Antibodies against native proteins

Rheumatoid factor is defined as an antibody that binds the Fragment crystallizable (Fc) portion of another antibody (IgG). RF and IgG form large ICs thought to contribute to pathogenesis. RF is not specific for RA as it is detected in other autoimmune diseases, systemic infections, and up to 10% of healthy individuals (Carson et al., 1981; Franklin et al., 1957; Nell et al., 2005; Sutton et al., 2000), showing that additional events are necessary to establish pathogenic autoimmunity. The mechanism behind the relatively common occurrence of RF in autoimmune disease is likely to include a positive selection for B cells recognizing Fc-rich ICs (Davidson and Diamond, 2001; Roosnek and Lanzavecchia, 1991). Pathogenic RF undergoes affinity maturation and somatic hypermutation, suggesting that T cells are involved in the process (Bugatti et al., 2007).

Glucose-6-phosphate isomerase (GPI) is a glycolytic enzyme in the cytoplasm of all cells. The articular cavity is lined with extracellular GPI for unknown reasons, and antibodies against GPI cause arthritis in mice (Ji et al., 2002; Matsumoto et al., 2002). Around 15% of RA patients have GPI antibodies, which is associated with higher disease activity (Matsumoto et al., 2003). Again, the occurrence of this antibody is not specific for RA, since other rheumatic diseases show similar prevalence.

Collagen type II (CII) is the major protein in joint cartilage and anti-CII antibodies from patients can transfer arthritis to naïve mice (Wooley et al., 1984). Antibodies against collagen type II are commonly present in patients around onset of the disease (Cook et al., 1996; Holmdahl et al., 1993; Mullazehi et al., 2012). Reported prevalence in patient populations varies from a few percent up to 60% (Cook et al., 1996; Lindh et al., 2014; Mullazehi et al., 2012), depending on type of assay and/or target epitopes used. The role of anti-CII immunity in initiating arthritis is well characterized in several species, targeting conserved epitopes on
the CII protein (Burkhardt et al., 2006; Lindh et al., 2014; Nandakumar and Holmdahl, 2006). It is also one of the few common targets in RA with joint specificity, making these autoantibodies interesting for studying the effector phase in arthritis (Nandakumar and Holmdahl, 2006), which is described further below. In paper III, the role of anti-CII antibodies in inducing nociceptive signaling is studied.

1.1.4.2 Antibodies against citrullinated proteins

Citrullination is an example of post-translational modification (PTM) of proteins, which is a common occurrence and involves enzymatic modification of a protein after its biosynthesis (Fig. 2). New functional groups or molecules are formed or added, like phosphate, amide, carbohydrate, or lipid. This changes the functional properties of the protein and can activate/deactivate enzymes. Citrullination, also called deamination, is the conversion of the amino acid arginine (positive) to citrulline (neutral), which is performed by peptidyl arginine deiminases (PADs) (Vossenaar et al., 2003) in the presence of high concentrations of Ca$^{2+}$. This changes the properties of the protein but the physiological function of this modification is incompletely understood. However, it seems that it renders the protein more immunogenic. PAD enzymes and citrullinated proteins have not been shown to be expressed in the thymus, so T cells with high affinity to citrullinated proteins would not be negatively selected and may enter peripheral tissues with citrulline specificity (Klareskog et al., 2013b).

**Figure 2. Representation of citrullination.** Citrulline is formed by a post-translational calcium-dependent enzymatic reaction mediated by peptidylarginine deiminase (PAD). Modified from (Cerqueira et al., 2013).

Antibodies against citrullinated proteins in RA were first demonstrated at the end of the 1990s (Girbal-Neuhauser et al., 1999; Schellekens et al., 1998), which led to development of standardized assays for detection in patients and continued research. It became apparent that ACPA is present in 60-70% of RA patients and that it is highly specific for RA, the prevalence is very low in the general population and in other rheumatic conditions (Schellekens et al., 2000; van Gaalen et al., 2004). The targeted citrullinated epitopes are located on a variety of proteins, including fibrinogen, vimentin, CII, and a-enolase (Klareskog et al., 2013b), and recent studies show that the antibodies are inherently cross-
reactive (Amara et al., 2013). An interesting feature of anti-citrulline immunity has come from studies using longitudinal serum samples from blood donors and patients. The occurrence of ACPA is seen several years before the onset of symptoms and diagnosis (Nielen et al., 2004; Rantapää-Dahlqvist et al., 2003). During this period the frequency of ACPA types, number of fine specificities, titers, and affinity remain low, until a period 6-12 month before onset of clinical joint symptoms where the antibody characteristics change into high frequency, high number of specificities, high titer, and high affinity (Brink et al., 2013; Sokolove et al., 2012; Suwannalai et al., 2012; van de Stadt et al., 2011a). Importantly, arthralgia is often apparent in the patients before onset of other clinical symptoms (Bos et al., 2010; van de Stadt et al., 2011b), suggesting that nociceptive signaling starts very early in the disease, in the pre-RA phase (Fig. 3).

Figure 3. A three stage model for ACPA-positive RA. Stage 1, the immune response: Environmental risk factors, such as smoking, may induce citrullination of proteins in the lungs. Altered antigen uptake, processing, and presentation of citrullinated antigens could in genetically susceptible individuals (like HLA-DR SE positive) lead to production of ACPA. At this stage pain is apparent in many individuals. Stage 2, the pathological inflammatory response: Unspecific arthritis, accompanied by citrullination of proteins in the joints. Recruitment of ACPA from the circulation results in the formation of ICs. Stage 3, chronic RA: Generation of citrullinated proteins, influx of immune cells, and production of cytokines and autoantibodies, as a result of the immune complex formation, convert the joint inflammation into chronic RA. Adapted from (Klareskog et al., 2008).

1.1.4.3 Effectors mechanisms of antibodies

Antibodies, also called immunoglobulins (Ig), are the major effector molecules of adaptive immunity. Antibodies can be grouped into five different isotypes; IgG, IgM, IgA, and IgE, which have different functions and distributions. The most common isotype in the circulation, both in normal condition and during disease, is IgG (75% of total Igs), and I am focusing on IgG in this thesis. The antigen-binding region (Fab) of the antibody is a hypervariable region
capable of binding three-dimensional epitopes with high affinity (Fig. 4). The defense against microbes and modulation of immune function is done through a variety of mechanisms that always include binding of the Fab-region to the antigen. This can neutralize the function of toxins or viral particles, and help the body to clear the specific factor. Furthermore, the Fc-part of the antibody activates the immune system, as macrophages and neutrophils will recognize antibodies bound to antigen and the target is eliminated by phagocytosis or antibody-dependent cellular cytotoxicity (ADCC) by NK cells. One or more antibodies bound to an antigen is called an immune complex (IC), and these creates a locally high concentration of antibodies, which augments the effector functions. The ICs can activate the complement cascade through the classical pathway, which involves Fc binding and cleavage of C1. These cleavage products initiate a cascade leading to lysis of target membranes (by membrane attack complex, MAC (C5b-C9)) and recruitment of immune cells (by C3a and C5a).

Formation of ICs leads to a conformational change in the Fc part of the antibody, which enables it to associate with Fcγ receptors (FcγRs). Importantly, this binding is dependent on a glycan located at Asn297 on the Fc (Fig. 4). The presence of the glycan is a requirement for IgG activation of FcγR, as deglycosylation of IgG prevents the Fc-FcγR interaction (Anthony et al., 2012; Nandakumar et al., 2013). The Fc – FcγR interaction has low affinity but high avidity, meaning that complex formation is necessary for potent activation.

In mice there are four types FcγRs; FcγRI, FcγRIIb, FcγRIII and FcγRIV, with similar human orthologs (Fig. 5) FcγRs are coupled to intracellular tyrosine kinases and can be either activating or inhibitory depending on the motif. They are widely expressed on hematopoietic cells with some cell type specificity, monocytes and macrophages express all receptors while B cells only express the inhibitory FcγRIIb (Nimmerjahn and Ravetch, 2008). Activating FcγRI, III and IV have immunoreceptor tyrosine-based activation motifs (ITAM) that causes

![Figure 4. Structure of IgG.](image-url)
the spleen tyrosine kinase (SYK) – phosphoinositide 3-kinase (PI3K) – phospholipase C (PLC) pathway to increase intracellular calcium levels and triggering of further downstream events (Odin et al., 1991). The inhibitory FcγRIIBs have immunoreceptor tyrosine-based inhibitory motifs (ITIM) and activation causes recruitment of phosphatases that leads to inhibition of PLC (Bolland and Ravetch, 1999), thus inhibiting ITAM-induced intracellular calcium increases.

**Figure 5. The family of Fc receptors for IgG.** Human and mouse FcγRs can be distinguished by their affinity for the antibody Fc-fragment and by the signaling pathways they induce. Mice and humans have one high-affinity receptor, FcγRI. All other FcRs have low to medium affinity. There is one single-chain inhibitory receptor, FcγRIIB, which contains an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytoplasmic domain. With the exception of human FcγRIIA and FcγRIIC, an activating FcR usually consists of a ligand-binding α-chain and a signal transducing γ-chain dimer, which carry immunoreceptor tyrosine based activating motifs (ITAM). A variety of human FcγR alleles with altered functionality exists, for example FcγRIIA131H have higher affinity to certain IgG subclasses compared to their allelic counterparts. Adapted from (Nimmerjahn and Ravetch, 2008).

Much of the information on FcγRs comes from studies of immune cells. However, several recent studies have shown expression and function of FcγRs in both central and peripheral neurons (Okun et al., 2010). Neuronal FcγRIIB is involved in cerebellar function and Alzheimer pathology of the hippocampus (Kam et al., 2013; Nakamura et al., 2007). In the periphery, motor neurons have been shown to take up ICs in their terminals, a process mediated by FcγRs that causes increases in intracellular calcium (Mohamed et al., 2002). The first evidence relating to sensory neurons came from *in vitro* studies using dissociated mouse
DRGs, which showed that IC triggering increased calcium and release of substance P (Andoh and Kuraishi, 2004). This was later repeated in cultures from rats, implicating TRPC3 as a key molecular target for the excitatory effect (Qu et al., 2012; 2011). So far, no studies have explored the contribution of FcγR to *in vivo* pain-behavior, which is the focus of Paper III.

### 1.2 PAIN IN RA

The primary goal of the treatment of patients with RA is to maximize long-term, health-related quality of life through control of symptoms, prevention of structural damage, and normalization of function and social participation (Smolen et al., 2010). Thus, reducing pain is one of the most important aims of therapy. Additionally, RA patients often rate pain as one of their most significant problems (da Silva et al., 2010; Heiberg and Kvien, 2002). Unfortunately, chronic pain in patients with RA remains a major clinical problem, even when the disease is under control or even in remission (Lee et al., 2011; Welsing et al., 2005). The pain reported varies between individuals and can occur upon mechanical stimulation of the joint, during movement, or spontaneously while at rest. When asked to describe the pain, patients with active disease often use words like shooting, throbbing, and sharp, while less-active disease is often described as tender, dullness, and gnawing (Burckhardt, 1984; Roche et al., 2003). Interestingly, many RA patients also report pain characteristics that are associated with neuropathic (nerve-injury) pain, like burning pain on touch and sudden electric shock-like attacks (Harden, 2005). This suggests that the nature of pain in RA is more complex than that solely due to inflammation. In fact, there is poor correlation between disease activity and pain (Altawil et al., 2016; Koop et al., 2015).

#### 1.2.1 The nociceptive system

Primary sensory neurons relay information about our internal and external environments from tissues to the brain, through the spinal cord or trigeminal system, allowing us to perceive noxious and non-noxious stimuli (Fig. 6). Pain, per definition, is an unpleasant sensory and emotional experience, which is often dependent on nociceptive input from the periphery. The sensory neurons have highly specialized adaptations to respond to a variety of mechanical, thermal, and chemical inputs. The heterogeneity in responses is mediated by differences in restricted localization, and expression of receptors, neuropeptides, and ion channels.

It is important to note the difference between acute and chronic pain. Acute tissue insults such as heat, cold, chemical or mechanical injury stimulate the nociceptors (pain receptors), located at the endings of the nociceptive primary neurons. The ability to detect and rapidly respond to noxious stimuli is vital for the survival of any organism. Pain causes advantageous behavioral changes, such as withdrawal of a limb from a flame or by reducing the use of a broken foot (Fig. 6). The modified behavior will prevent tissue damage or allow healing. Withdrawal from the acute stimulus and full tissue recovery should result in restoration of homeostasis and end the activity in the pain pathways. However, continuous or repetitive nociceptive stimulation can lead to a series of pathophysiological changes in pain...
processing such that the pain signaling outlives its usefulness as a warning system, and the pain becomes chronic and debilitating. This shift from acute to chronic pain is mediated via complex changes initiated and maintained at both peripheral and central locations. This implicates that pain is not generated by a static, hardwired system, but instead results from the summation effect of highly plastic mechanisms and circuits.

Figure 6. Nociceptive pathway and withdrawal reflex. Noxious stimuli are sensed by nociceptors that signal to the spinal cord, where the afferents synapse with projection neurons that send signals to the brain where the pain is perceived. Additional synapse is made from the primary afferent to interneurons, which activate flexor motor neurons to elicit withdrawal of the affected limb. Adapted from (Talbot et al., 2016).

1.2.1.1 Nociceptor classification

Nociceptor are peripheral sensory nerve fibers that detect noxious stimuli, suggesting molecular or biophysical properties to selectively respond to potentially damaging stimuli. Their cell bodies are located in the dorsal root ganglia (DRG) for the body and trigeminal ganglion (TG) for the face, and have branches innervating both the target organ and the dorsal horn of the spinal cord or the spinal nucleus of the trigeminal complex. Nociceptors are very heterogeneous and can be broadly classified according to fiber diameter and degree of myelination, which determines conduction speed. Generally, medium diameter myelinated afferents (Aδ) mediate fast, localized pain, while small diameter, unmyelinated C-fibers signal poorly localized, slow pain. The large diameter myelinated fibers (Aβ) are often associated with fast conducting low-threshold mechanoreceptors, but a substantial proportion of A-fiber nociceptors are Aβ-type (Djouhri and Lawson, 2004; Schaible et al., 2009). Aδ and C-fibers can be further subdivided into sub-classes. Type I Aδ include high threshold mechanical (HTM) nociceptors that respond to both mechanical, chemical stimuli but with a high heat threshold (above 50°C). Type II Aδ nociceptors have a lower heat threshold but a
much higher mechanical threshold. The C-fibers are highly heterogenous and most are polymodal (Fig. 7), often referred to as heat and mechanically sensitive, while other respond to mechano-cold or just heat. One population of unnyelinated C-fibers, common in joints, are called “silent nociceptors” as they are unresponsive to mechanical stimulation during normal conditions but become responsive after e.g. an injury, and likely contribute largely to both initiation and maintenance of hypersensitive states (Basbaum et al., 2009; Schaible and Schmidt, 1985). Glutamate is the primary neurotransmitter for all nociceptors, while subtypes of C-fibers also synthesise peptides like substance P and calcitonin gene-related peptide (CGRP). These peptidergic fibers often express the NGF-receptor TrkA. The nonpeptidergic C-fibers express the IB4 isolecitin, as well as purinergic receptors of the P2X types (Basbaum et al., 2009). The variety of nociceptor functional and molecular subclasses associate with specific detection of pain modalities.

1.2.1.2 Signal initiation and transduction

The primary efferent nerve terminal detects environmental stimuli, converts them into changes in membrane potential and summates them. If the resulting generator potential, or depolarization, is sufficient, it is transformed into action potentials and the signal is propagated along the nerve to the dorsal horn. The detection in the terminal is done in different ways depending on the receptor type, which determines if the fiber is nociceptive or not. Sensory neurons express at least three classes of surface proteins that affect signal initiation and transduction: ion channels, metabotropic G protein-coupled receptors (GPCRs), and receptors for neurotrophins and cytokines (Fig. 7).
Figure 7. Heterogeneity of nociceptors. One way of classifying nociceptors is by the response profile of the afferent. Illustration of a sensory nerve with peripheral terminal to the left and central terminal to the right. Example of a polymodal afferent that responds to mechanical, thermal and chemical stimuli, due to various receptors on the same afferent. Voltage-gated channels along the nerve propagate the signal to the central terminal in the dorsal horn, where several ion channels and receptors modify transmitter release. Adapted from (Gold and Gebhart, 2010).

Nociceptors express a wide variety of ligand gated ion channels that responds to stimuli and can rapidly change the membrane potential. One of the most studied class is the TRP channels, which have a high permeability to calcium and are multimodal receptors (Julius, 2013). TRPV1 defines a population of nerve fibers that are activated by noxious heat (above 43°C), low pH (5.2), capsaicin, and certain lipids. TRPM8 is in the same receptor class but detects innocuous and noxious cold, as well as cooling agents such as menthol (Julius, 2013; Takashima et al., 2010). Another family of voltage-independent channel are acid sensing channels (ASICs). They are activated by acidic pH and when activated are permeable to Na\(^+\) causing depolarization and secondary accumulation of Ca\(^{2+}\). Nerves expressing ASICs primarily innervate muscle, joint and bone (Wemmie et al., 2013). To detect mechanical sensation, the general consensus is that pressure opens a mechanosensitive cation channel to elicit rapid depolarization (Basbaum et al., 2009). However the molecular basis of mechanotransduction is far from clarified. It is a clinically highly relevant subject since mechanical hypersensitivity is a major problem for many pain patients. Several types of ion channels have been implicated in mechanotransduction, including TRP, DEG/EnaC (ASICs), and Piezos, but their exact role in nociceptive signaling remains illusive (Basbaum et al., 2009; Ranade et al., 2015).

There is a unique repertoire of voltage gated ion channels that are vital for controlling the excitability of the nociceptor and propagation of the signal. The key determinants of excitability are Na\(^+\) channels, like Na\(_v\)1.7, Na\(_v\)1.8, and Na\(_v\)1.9, which have some differences in localization and receptor properties. For example, Na\(_v\)1.8 has a higher threshold activation than Na\(_v\)1.7, but carries most of the current underlying the depolarization phase of the action potential in C-type neurons (Benarroch, 2015; Renganathan et al., 2001). Na\(_v\)s are the target...
of local anesthetics, such as lidocaine, which effectively blocks the propogation of nociceptor signaling. Nociceptors express several subtypes of \( K^+ \) channels, including \( K_v \), that dampen the excitability of the nerve by regulating membrane potential, action potential threshold, as well as shape, firing frequency and adaptation (Tsantoulas and McMahon, 2014). Nociceptors in addition express several types of voltage-gated \( Ca^{2+} \) channels of L-, N-, and T-type. The \( \alpha_2\delta \) subunit of high voltage gated \( Ca^{2+} \) channels, including the N-type channels, is of particular interest. This subunit is upregulated in models of neuropathic and inflammatory pain, and is the target of the analgesics gabapentin and pregabalin (Bauer et al., 2010; Davies et al., 2007; Su et al., 2015).

\textit{G protein-coupled receptors} are cell surface proteins that are ligand activated and initiate intracellular signaling pathways that can affect the properties of the neuron by regulating excitability. They are often used to define the histological and functional identity of sensory neurons. Excitatory receptors for bradykinin, protease activated receptors, and prostaglandin E2 receptors, activate G-coupled adynelate cyclase – cAMP – PKA or PLC – DAG – PKC pathways that have a wide variety of intracellular effects, for example sensitization and activation of TRPV1 (Gold and Gebhart, 2010; Schaible et al., 2002). The are also examples of inhibitory GPCRs on nociceptors, including the \( \mu, \kappa, \text{and } \delta \) opioid receptors (Mousa, 2003). Activation of the opioid receptors will reduce intracellular cAMP, activate \( K_v \), and inhibit \( Ca_v \), potently reducing excitability of the neuron.

The neurotrophin NGF is required for survival and development of sensory neurons during embryogenesis, but is also produced during tissue injury and can directly activate peptidergic C-fibers producing hypersensitivity (Lewin and Mendell, 1993). The neurons express the high-affinity receptor TrkA but also the low-affinity receptor p75, and activation by NGF causes a rapid increase in TRPV1 activity. Additionally, the NGF protein is also internalized and transported to the nucleus, promoting expression of pronocicepve peptides and proteins like substance P, TRPV1, and Nav1.8 (Basbaum et al., 2009; Chao et al., 2003; Snider and McMahon, 1998).

\textit{Cytokines and chemokines} are an integral part of the inflammatory cascade, released from variety of celltypes in response to injury or infection. Several of the receptors for both cytokines and chemokines are present on nociceptors, and there is evidence for a direct involvment of them in the regulation of hypersensitivity in a number of experimental models of pain, as well as in humans (Abbadie et al., 2009; Ren and Dubner, 2010; Schaible et al., 2010). Of relevance for this thesis (paper III), the chemokine IL-8 (mouse analogue CXCL1), a well-characterized chemo-attractant, directly activates or sensitizes nociceptors by acting on its receptor CXCR2, which is present on sensory neurons (Qin et al., 2005; Wang et al., 2008; Zhang et al., 2013). The receptor activation modify \( Na_v \) and \( K_v \), as well as TRPV1 function, to increase sensitization and excitability in sensory neurons (Dong et al., 2012; Yang et al., 2009).
1.2.1.3 Neurobiology of bone and joint

The joint, ligaments, fibrous capsule, meniscus, periosteum, synovial layer, and surrounding bone structures are innervated by sensory nerves (Aβ, Aδ- and C-fibers) and noxious sensation can be evoked from these structures by various factors (Schaible et al., 2009). Many of these fibers are silent nociceptors, i.e. nerve fibers that are only activated by injury or damage to the structures in connection with inflammation. Once active, these fibers can either be spontaneously active or have reduced thresholds such that previously ineffective stimuli, in either noxious or innocuous range, can evoke activity. In contrast to the skin, the majority of the sensory neurons innervating bone and joint are thinly myelinated TrkA+ and/or peptide rich CGRP fibers, with minor innervation of Aβ and TrkA− peptide poor fibers (Fig. 8) (Jimenez-Andrade et al., 2010; Zylka et al., 2005). In the bone marrow and cortical bone, the nerve fibers most often co-localize with blood vessels, while the periosteum is densely innervated in a grid pattern (Martin et al., 2007). Interestingly, the articular cartilage of the joint lack innervation of sensory nerve fibers, so pain resulting from damage to cartilage itself is likely initiated in adjacent structures, like subchondral bone and synovium (Mantyh, 2014; Schaible et al., 2009). This data underlines the importance of knowing the microenvironment of the disease process, not just for modeling in animal studies but also for choosing pharmacological targets in patients.

Figure 8. Types of sensory nerve fibers innervating bone. Schematic illustration of the organization and pattern of innervation of the bone. The types of sensory neurons that innervate the bone are unmyelinated C fibers and thinly myelinated Aβ fibers. The relative density of sensory fibers is greatest in the periosteum, followed by bone marrow and then cortical bone (A). Primary afferent neurons innervating bone have their cell bodies in the DRG and project to the spinal cord. The great majority (>80%) of the bone innervating sensory fibers express TrkA (the receptor for NGF), while <30% of the nerve fibers that innervate the skin express TrkA (B). Adapted from (Mantyh, 2014).
1.2.1.4  Peripheral and central sensitization

Inflammation or injury such as fracture is characterized not just by activation of the nociceptive system but also by increased sensitivity to stimulation, a process that starts within minutes and can persist for months (Schaible et al., 2009). This is caused by several factors, described above, that cause altered activation thresholds of nociceptors, giving rise to hyperalgesia (increased pain intensity in response to a normal painful stimulus) and allodynia (pain due to a stimulus that does not normally provoke pain). Sensitization also includes an increase of suprathreshold response, spontaneous discharges, and increases in receptive field size of the nociceptive neurons (Grigg et al., 1986; McDougall, 2006; Schaible and Schmidt, 1985).

During persistent input, several changes also take place at the central level (dorsal horn) that amplify the nociceptive signal. It is a complex process that involves many types of cells and circuits, involving augmenting glutamatergic transmission and activation of glia cells (Tsuda et al., 2003; Woolf, 1983; Woolf and Salter, 2000). Interestingly, several of the molecules released in the periphery during inflammation, like TNF and IL-6, are also released by glia during activation, contributing to the central sensitization. In addition to enhancing the peripheral input, central sensitization contributes to the painful sensation around the original site. This secondary hyperalgesia involves heterosynaptic facilitation, where normally innocuous inputs, for example Aβ efferents normally responding to light touch, now signal into nociceptive circuits producing mechanical allodynia (Basbaum et al., 2009; Campbell et al., 1988). Additionally, factors released during dorsal horn activity can cause inhibitory neurotransmitters to depolarize rather than hyperpolarize neurons, further amplifying the nociceptive signaling (Coull et al., 2005). To prevent establishment of a hyperexcitable nociceptive pathway, it is important to understand how the peripheral signal originates, which is the main focus of this thesis.

1.2.2  Pharmacological pain management in RA

There are several pharmacological options for treating pain in RA, both direct acting analgesics and indirectly reducing the disease activity and inflammation. Unfortunately, despite optimal disease control, pain can remain a major problem (Altawil et al., 2016; Lee et al., 2011; McWilliams et al., 2012).

Suppression of joint inflammation and associated pain can be achieved using a variety of drugs. Traditional treatments for arthritis like glucocorticoids and methotrexate have documented beneficial effect on pain by reducing synovial inflammation (Kirwan and Boers, 2004; Williams et al., 1985). Due to side effects, steroid use is limited and symptoms may return after discontinued use, while methotrexate is often better tolerated for chronic treatment. Biologic disease modifying anti-rheumatic drugs (DMARDs), like cytokine blockers, also reduce joint inflammation and pain (Keystone et al., 2004) and can have an additive effect on pain relief when administered as co-therapy with methotrexate (Weinblatt
et al., 2006). Since inflammatory cytokines such as TNF and IL-6, can directly affect peripheral nociceptive neurons (Brenn et al., 2007; Sommer and Kress, 2004), part of the analgesic effect of patients experience is likely due to inhibiting the direct effect of the cytokines. In fact, analgesic effect from the TNF-blocker was reported to occur well before any reduction in inflammation (Rech et al., 2013). Similar results has also been reported in animal models of arthritis (Boettger et al., 2008; Hess et al., 2011).

There are several types of analgesic drugs used in RA for pain management, for example non-steroid anti-inflammatory drugs (NSAIDs), paracetamol, opioids and opioid-like drugs, and neuromodulators (anticonvulsants, antidepressants, and muscle relaxants). However, the number of well-performed clinical trials focusing on pain is limited, and the trials done show limited efficacy, especially beyond 6 weeks (Walsh and McWilliams, 2014). Long-term use also increases risks of side effects, making analgesic treatment a constant balance between the risk and benefit for the patient and often limiting its use (Walsh and McWilliams, 2014; Whittle et al., 2012). Additionally, there are few clinical studies on the safety of using analgesic in patients with comorbidities like gastrointestinal or cardiovascular disease (Radner et al., 2012). Combining different classes of analgesics can sometimes have a synergistic effect, thus achieving better pain control than with monotherapy, although with possible increased risk of side effects. A recent review of pain management in neuropathic pain showed evidence of improvement in pain control with combination therapy (Finnerup et al., 2010). Unfortunately, there is insufficient evidence from clinical trials in RA to make similar recommendations (Ramiro et al., 2011). Despite the limitations of analgesics, it is important that patients can decide their treatment based on informed choices and individual preference. It is also important to thoroughly study which pain mechanisms are active in patients to make recommendations for treatments. For example, pain in patients without an inflammatory component are unlikely to get relief from NSAIDS.

1.3 EXPERIMENTAL ARTHRITIS

Since many factors are unknown in the pathogenesis of human RA, modeling the disease in animals is difficult. Frequently arthritis pain studies involve injection of a substance that causes inflammation, like carrageenan or complete Freund’s adjuvant (CFA). These substances produce robust inflammation and subsequent pain behavior in rodents but are relatively short lasting, from a few hours up to 1-2 weeks. Injection of these substances into the joint cavity causes infiltration of immune cells and synovial hypertrophy but other aspect of RA, like bone erosion and cartilage erosion, are usually not present. Furthermore, RA is a systemic polyarthritis involving both innate and adaptive immune system, which may impact on how the sensory neurons in the joint are activated. Thus, monoarthritic models that activate predominately the innate immune system might not be the optimal way of studying pain in RA.
To enable studies to involve the complex pathology of RA, models involving immunization are sometimes used. This comprises inoculation with an antigen and adjuvant, causing breakage of immunological tolerance and a T- and B-cell driven reaction against the antigen. A widely used antigen is CII, called the collagen induced arthritis model (CIA) (Courtenay et al., 1980; Trentham et al., 1977). Inoculation with CII triggers the immune system to produce anti-CII antibodies leading to a chronic polyarthritis with RA-like joint pathology. CIA is generally considered a good model with features such as breaking of self-tolerance, targeted cartilage immunity, and T- and B-cell activity (Holmdahl et al., 1990; Wooley et al., 1981), that have similar MHC II genetic association as human RA (Gregersen et al., 1987; Wooley et al., 1981). The CIA model results in robust and long-lasting pain behavior in rodents and has been used in several pain studies (Baek et al., 2005; Broom et al., 2008; Clark et al., 2012; Inglis et al., 2007; Kinsey et al., 2011; Nieto et al., 2015; Patro et al., 2011). A drawback with the model is the progressive nature of the arthritis. Once initiated the inflammation and bone destruction will progress, not reflecting the human disease course and making it impractical for long-term behavior studies.

1.3.1 Collagen antibody-induced arthritis

The strong association of anti-CII antibodies and disease in CIA led to the development of the collagen antibody induced arthritis (CAIA) animal model (Nandakumar et al., 2003; Stuart and Dixon, 1983; L. Svensson et al., 1998; Terato et al., 1992), which Paper III explores from a sensory perspective. In the CAIA model the breaking of tolerance against CII is bypassed, reflecting the antibody-mediated effector phase of RA. It is induced by an intravenous or intraperitoneal injection of a mixture of anti-CII antibodies of IgG2a and IgG2b isotype. Lipopolysaccharide (LPS) is injected systemically 3-5 days after injection of CII antibodies in order to synchronize onset of joint inflammation and enhance disease severity and incidence by toll-like receptor 4 (TLR4) mediated activation of complement components and increased release of inflammatory mediators (Nandakumar et al., 2003; Terato et al., 1992). A polyarthritis resembling active CIA pathology develops, though with a faster onset (5-7 days), resulting in a transient inflammation lasting 3-4 weeks. Primarily small joints in the front and hind paws are affected but occasionally also knee and vertebral joints are involved. CAIA susceptibility is MHC independent and is able to manifest in strains resistant to CIA (Nandakumar et al., 2003; Stuart and Dixon, 1983). Mouse strains like DBA/1, B10.RIII, Balb/C and CBA, have high susceptibility (>90% incidence) while C57BL/6, B10.Q and NOD.Q have low susceptibility (<50% incidence), indicating a genetic dependence in incidence and severity (Nandakumar and Holmdahl, 2007). Mice lacking activating FcγRs (Kagari et al., 2003) or C5a signaling (Grant et al., 2002; Watson et al., 1987) are resistant to CAIA. Interestingly, such mice still accumulate IgG and C3 on the cartilage surface but inflammation is not initiated (Grant et al., 2002), highlighting the importance of the innate immune system in the effector phase. The CAIA model is a tool to study the effector phase of arthritis but does not involve T and B cells that are part of the normal RA pathophysiology. The transient inflammation gives a good opportunity to study several phases of an RA flare (including pre- and post-inflammation). Interestingly, there is a
disconnection between inflammation and the pain behavior, as mechanical hypersensitivity is apparent before onset of inflammation (Agalave and C. I. Svensson, 2014; Bas et al., 2012). Paper III focuses on the role of collagen antibodies in the initiation of pain behavior in the pre-inflammatory phase.
2 AIMS OF THESIS

The overall aim of the thesis is to explore mechanisms that drive initiation and maintenance of nociceptive signaling in autoimmune disease. Specifically how antibodies that are common in rheumatoid arthritis patients affect and interact with peripheral sensory neurons, in structures that are targeted in the disease: i.e. joint and bone. The thesis has two specific aims:

1. To characterize the *in vivo* consequences of anti-citrulline immunity and identify the effects on nociceptive processing.

2. To investigate the pain-like behavior in early phase of the CAIA model, with specific focus on the role of anti-CII antibodies in the initiation of sensory signaling.
3 MATERIAL AND METHODS

3.1 ANIMAL MODELS

3.1.1 Animals

Animal experiments were approved by the local ethics committee for animal experiments in Sweden. Mice were housed in standard cages (3-5 per cage) in a climate controlled environment maintaining a 12 h light/dark cycle with access to food and water ad libitum. Several strains of mice have been used for the work in this thesis. For Paper I and II Balb/C and B10.RIII were used. For Paper III B10.RIII, B10.Q, and Balb/C were used. Additionally, several genetically modified mice were also used in Paper III: B10.Q C5\(^{-/-}\), which are mice with a natural complement component 5 mutation leading to a complete deficiency; B10Q.ACBB (Anti-C1 B-cell) (Cao et al., 2011) which is a germline encoded anti-CII B cell knock-in strain with spontaneous production of anti-CII IgG; ACB C9\(^{-/-}\), which are ACB mice lacking cartilage matrix protein collagen type IX (C9); Fc\(\gamma\) chain \(^{-/-}\), which are mice lacking the common \(\gamma\)-chain and thus, have no functional activating Fc\(\gamma\)Rs.

3.1.2 Injection of autoantibodies

3.1.2.1 ACPA

Human and mouse antibodies that bind to citrullinated epitopes were used in paper I and II, as a novel model of ACPA-induced pain behavior. Mice were injected i.v. with 0,125-4 mg of human IgG and 2 mg of murine IgG in 100-150 µl saline. The donors were patients visiting the Rheumatology clinic at Karolinska University Hospital, who fulfilled the ACR/EULAR criteria for RA. They were tested for anti-CCP2 reactivity and samples were collected from ACPA\(^+\) patients, ACPA\(^-\) patients, and also healthy controls. Plasma, sera, and synovial fluid were collected and kept at -80°C until processed. To purify the collected human antibodies, samples were centrifuged at 3000 g for 5 minutes and diluted 1:5 (v/v) in PBS. IgGs were purified from diluted plasma and sera on HiTrap Protein G HP columns. Eluted IgGs were dialyzed against PBS and the antibodies from ACPA\(^-\) RA patients were applied to the CCP2 affinity column. ACPAs were eluted using 0.1 M glycine–HCl buffer (pH 2.7) and the pH was directly adjusted to 7.4 using 1 M Tris (pH 9). IgGs not binding to the CCP2-column were used as control in control experiments, they were denoted as flow through (FT). Autoantibodies were concentrated and the buffer exchanged to PBS using the 10 kDa Microsep™ UF Centrifugal Device. Recovery and purity of total ACPAs were analysed by SDS-PAGE followed by Coomassie Blue staining and anti-CCP2 reactivity. The concentration (mg/ml) of total IgG was calculated based on the initial plasma/sera volume applied to the Protein G column and the amount of IgG eluted from the column. The endotoxin levels were determined in the different pools of autoantibodies by the limulus amebocyte lysate assay and the cut-off for positivity was assumed as > 0.05 EU/ml. Three different ACPA pools were utilized for the in vivo experiments (paper I and paper II): ACPA pool 1 containing autoantibodies purified from 38 plasma samples, ACPA pool 2
containing autoantibodies from 6 plasma/sera samples and ACPA pool 3 that includes autoantibodies purified from 25 plasma/sera samples. To prepare the ACPA+ pool, antibodies isolated from the same plasma/sera samples as used for ACPA pool 2 were selected. This pool of antibodies was constituted by ACPA and non-ACPA IgGs.

Additionally, monoclonal ACPA were used in paper I and II. Production of murinized monoclonal antibodies D10, B2, C7 and E2 is described in detail in (Amara et al., 2013). In brief, single B-cells were sorted from synovial fluid of ACPA+ patients into a 96-well plate. Digested PCR products from single cells were cloned into expression vectors containing Igγ1, Igκ, or Igλ constant regions and transfected into human embryonic fibroblasts HEK293. Supernatants were collected and purified by binding to protein G-sepharose column and expression of heavy and light chain, as well as purity, was verified by PAGE. Reactivity of the generated monoclonal antibodies against citrullinated and native form of α-enolase (CEP-1), vimentin (aa 60-75), and fibrinogen (aa 36-52) peptides was determined with ELISAs. The E2 antibody (also derived from a RA synovial B cell) reacts against human tetanus and was detected using ELISA. Murinization of the human monoclonal antibodies was performed by replacing the full human IgG1 Fc by the murine IgG2a Fc. Mouse monoclonal ACC4 was produced in hybridoma generated from mice immunized with PAD4-treated CII. The generated antibody binds the citrullinated C1 epitope on CII as an α-chain peptide and interacts directly with citrulline as shown earlier with crystal structure (Uysal et al., 2009). It also cross-reacts with the cyclic citrullinated filaggrin peptide (CCP1), but not with non-citrullinated forms of CII.

3.1.2.2 Collagen type II antibodies

Our normal CAIA protocol consist of a lipopolysaccharide (LPS) injection 5 days after injection of antibodies, to induce inflammation. In Paper III, that protocol was used in one experiment. Subsequent experiments focused on the pre-inflammatory phase, so LPS was not injected. Mice were given i.v. injections on day 0 with either saline or anti-CII mouse monoclonal antibodies (mAbs) (4 mg in a total volume of 150 µl saline). Arthritogenic anti-CII mAbs; M2139, CIIC1, CIIC2, and UL1 (Nandakumar and Holmdahl, 2005) were injected either as a cocktail (1 mg/each) or as single antibodies (0.5-4 mg). In addition, the non-arthritogenic anti-CII mAb CIIF4 (Croxford et al., 2010; Nandakumar et al., 2008), as well as IgG2a and IgG2b isotype control mAb were used. Antibodies were produced and purified as described earlier (Nandakumar and Holmdahl, 2005). Lipopolysaccharide was used in one experiment and injected intraperitoneally (i.p.) 5 days after injection of anti-CII mAb cocktail.

In several experiments modified antibodies were used. To remove the N-linked glycans, M2139 mAb was incubated with recombinant endo-β-Nacetylglucosaminidase (EndoS) fused to glutathione S-transferase (GST) as previously described (Collin and Olsén, 2001). Briefly, GST-EndoS in phosphate buffer solution (PBS) was mixed with M2139 mAb and incubated at 37°C for 16 h. GST-EndoS was then removed using Glutathione-Sepharose 4B columns. Further purification of the antibodies was done using an ion exchange column. SDS/PAGE
and Lens culinaris agglutinin (LCA) lectin blotting were used to confirm complete removal of GST-EndoS and efficacy of EndoS cleavage. Fab fragments were prepared from the anti-CII mAb cocktail using Pierce Fab Preparation Kit according to the manufacturer’s instructions. Fab fragments and EndoS treated antibodies corresponding to 4 mg anti-CII mAb cocktail were injected i.v.

Table 2  Antibodies used in the thesis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
<th>Type</th>
<th>Other information</th>
</tr>
</thead>
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<td>Human Polyclonal IgG</td>
<td>(Ossipova et al., 2014)</td>
</tr>
<tr>
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<td>Non-CCP2</td>
<td>Human Polyclonal IgG</td>
<td>Control antibody (Ossipova et al., 2014)</td>
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<tr>
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<td>Control antibody</td>
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<td>Mouse IgG2a</td>
<td>(Amara et al., 2013)</td>
</tr>
<tr>
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<td>CEP-1, cit-Fib, cit-vim</td>
<td>Mouse IgG2a</td>
<td>(Amara et al., 2013)</td>
</tr>
<tr>
<td>1276:01:C07</td>
<td>CEP-1, cit-Fib, cit-vim</td>
<td>Mouse IgG2a</td>
<td>(Amara et al., 2013)</td>
</tr>
<tr>
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<td>Human tetanus</td>
<td>Mouse IgG2a</td>
<td>Control antibody (Amara et al., 2013)</td>
</tr>
<tr>
<td>M2139</td>
<td>J1 epitope on CII</td>
<td>Mouse IgG2b</td>
<td>(Nandakumar and Holmdahl, 2005)</td>
</tr>
<tr>
<td>CIIC1</td>
<td>C1 epitope on CII</td>
<td>Mouse IgG2a</td>
<td>(Nandakumar and Holmdahl, 2005)</td>
</tr>
<tr>
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<td>(Nandakumar and Holmdahl, 2005)</td>
</tr>
<tr>
<td>UL1</td>
<td>U1 epitope on CII</td>
<td>Mouse IgG2b</td>
<td>(Nandakumar and Holmdahl, 2005)</td>
</tr>
<tr>
<td>CIIF4</td>
<td>F epitope on CII</td>
<td>Mouse IgG2b</td>
<td>Anti-inflammatory (Nandakumar et al., 2008)</td>
</tr>
<tr>
<td>ACC4</td>
<td>Cit-C1 epitope on CII</td>
<td>Mouse IgG1</td>
<td>(Uysal et al., 2009)</td>
</tr>
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<td>IgG2a control</td>
<td>Human HLA-DRa</td>
<td>Mouse IgG2a</td>
<td>Control antibody</td>
</tr>
<tr>
<td>IgG2b control</td>
<td>Human parathyroid epithelial cells</td>
<td>Mouse IgG2b</td>
<td>Control antibody</td>
</tr>
</tbody>
</table>
3.1.3 Arthritis score and arthritis incidence

The development of arthritis in the fore and hind paws was monitored by visual inspection as described previously (Bas et al., 2012; Nandakumar et al., 2003). Briefly, visible signs of inflammation, defined as redness and swelling, were scored on a 0–60 scale by investigators blinded to the origin and treatment of the mice. Each inflamed digit was noted as 1 point and inflammation of the metacarpus/metatarsus and ankle joint as 5 points, giving a maximum of 15 points per paw. Incidence is calculated as percentage of mice that were positive for arthritis. Toes had to be inflamed at least two consecutive days to be defined as arthritic, to avoid false positives due to loss of nails etc.

3.1.4 Pharmacology

In Papers I and II, mice were treated with the CXCR1/2 antagonist reparixin (L-lysine salt), which was injected subcutaneously (s.c. in 100 µl saline) twice daily (30 mg/kg/day).

In Paper III mice were treated with the cyclic peptide C5a-receptor inhibitor (PMX53, s.c., 3 mg/kg in saline) 1 h prior to injection of anti-CII mAb cocktail and then once daily 3 h prior to assessment of mechanical hypersensitivity for 5 days.

3.1.5 Metalloprotease activity

Mice injected with either saline, 1 mg hACPA, or 4 mg anti-CII IgG received i.v. injection of MMPsense 680 (2 nmoles in 150 µl PBS/mouse) 24 h before sacrifice. Paws were removed and scanned in an Odyssey CLx (LI-COR) near-infrared system. The signal intensity was quantified and normalized to saline injected mice and the data presented as a heat map.

3.2 ASSESSMENT OF PAIN-LIKE BEHAVIOR

Mechanical and thermal sensitivity in the hind paws and reduced locomotion were used as measures of evoked and spontaneous pain-like behavior, respectively. Assessment of mechanical and thermal sensitivity was performed on indicated days and locomotion was assessed during the third night after antibody injection. Mice were allocated to have even baseline tactile thresholds across treatment groups. The investigators were blinded for the origin and treatment of the mice during behavioral assessments, until after analysis of the results.

3.2.1 Mechanical sensitivity

The mice were habituated in individual compartments on top of a wire-mesh surface (Ugo Basile) prior to experiment. On test days, mice were given time to acclimatize and then optiHair filaments of increasing buckling force (0.5, 1, 2, 4, 8, 16, and 32 mN) were applied to the plantar surface of the paw until the filament bent slightly. A brisk withdrawal of the paw within 2-3 seconds was noted as a positive response. A 50% withdrawal threshold was calculated using the Dixon up-down method (Chaplan et al., 1994) and results from both hind paws were averaged and presented as % of baseline values. After unilateral intra-articular injections only the result from the ipsilateral paw was used. In addition to presenting the results as 50% withdrawal threshold in Paper I, data were also presented as an algesic
index, a calculation that defines the effect of reparixin treatment. It represents the area (based on withdrawal threshold in percent and time in days) between the extrapolated line from start of treatment (day 6) and the time–response curve after reparixin or saline injection. Increasing values indicate decreasing hypersensitivity.

### 3.2.2 Heat sensitivity

Heat sensitivity was examined using a modified Hargreaves box (Dirig et al., 1997). Mice were placed individually in Plexiglas cubicles on the glass surface and allowed to habituate. A radiant heat stimulus, which was a fixed distance beneath the glass, was aimed at the selected hind paw until a motion sensor detected a brisk withdrawal, which ended the stimulus. Elapsed time is automatically recorded with a cutoff at 20 s to prevent tissue damage. Three measurements from each paw were averaged and presented as latency (in seconds) of the withdrawal.

### 3.2.3 Cold sensitivity

To assess sensitivity to cold, the 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, and licking the paw) was recorded. The test was repeated three times on each paw, with at least 5 min between tests, and the average was calculated.

### 3.2.4 Locomotion

Food and water consumption, and activity level of the mice during a full night cycle was measured using an Oxymax/Comprehensive Lab Monitoring System (CLAMS). Mice were habituated for 24h in individual testing cages before being moved into the CLAMS just before the start of the night cycle (18:00-06:00). Infrared sensors detected movement in X, Y and Z-axes and recorded number of beam breaks during the testing period. These values were then summed to show total movement over the whole 12 h night cycle. A feeder system connected to a scale and automated water device recorded consumption during the period. The data is presented as total movement (total number of XY-axis beam breaks), ambulation (number of consecutive XY-axis beam breaks), rearing (number of beam breaks in the Z-axis), food intake (g), and water intake (ml)

### 3.3 CELL CULTURE

#### 3.3.1 Osteoclast cultures and chemokine analysis

For in vitro osteoclasts generation, bone marrow cells were obtained from wildtype Balb/c mice and CD-11b⁺ cells were then isolated using anti-CD-11b microbeads. CD-11b⁺ cells were seeded with 280x10⁵ cells per well in DMEM containing 10% heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin and stimulated with M-CSF 25 ng/ml and RANKL 25 ng/ml. From day 6, either saline, ACPA, or
FT was added to the media (1 µg/ml), which was replenished every two days with fresh supplements.

Osteoclasts were analyzed using Tartrate-resistant acid phosphatase (TRAP) staining with a leukocyte acid phosphatase kit 387A following manufacture instructions. TRAP positive cells with not less than 3 nuclei were counted manually as osteoclasts in the Nikon inverted light microscope.

Level of CXCL1 (KC-GRO) and CXCL2 (MIP-2α) was measured in the supernatants from the cultured cells and were analyzed using V-Plex immunoassay kit (Meso Scale Discovery) for CXCL1 and ELISA kit for CXCL2, diluted 1:2 in assay diluents and according to manufacturers protocol. Limit of quantification (LOQ) was 0.8 pg/ml for CXCL1 and 7.8 pg/ml for CXCL2.

### 3.3.2 DRG cell culture

In Paper I and III, several studies are based on in vitro studies on cultures DRG cells. The DRGs (L6-C1) from Balb/c mice were extracted and placed in ice-cold Dulbecco’s PBS until they were enzymatically dissociated with papain (1.7 mg/ml) (30 min at 37°C) followed by a collagenase I (2 mg/ml) and dispase II (8 mg/ml) enzyme mix (30 min at 37°C). The cells were then gently triturated in Leibovitz’s medium supplemented with 10% heat-inactivated bovine serum, 1% penicillin and streptomycin (Invitrogen) and 10 µM mitotic inhibitor (5-fluoro-2-deoxyuridine). The cell suspension was plated on uncoated well plates for 1.5-2 h before transferred to poly-D-lysine and laminin pre-coated well plates. The cells were maintained at 37°C in 5% CO2 atmosphere and the medium replaced after 24 h and then every third day. For functional experiments, immune complexes were applied to the cultures. Collagen II mAb immune complex (CII-IC) stock solution (1 mg/ml) was prepared by mixing anti-CII mAb cocktail (1 mg/ml) with rat CII (1 mg/ml, detailed in (Burkhardt et al., 2002), at a ratio of 1:1 at 37°C with gentle shaking for 30 min.

#### 3.3.2.1 CGRP release

After 6 days in culture, the medium was removed and the cells were washed twice with HEPES buffer (25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM dextrose, 0.1% (w/v) bovine serum albumin, pH 7.4 with NaOH) and placed in new HEPES buffer for 30 min at 37°C (pre-stimulation). The HEPES buffer was collected for analysis of basal CGRP release. The cells were then incubated with CII-IC (0.1, 1 and 10 µg/ml), anti-CII mAb cocktail, CII antigen, or control IgG2b (1 µg/ml) in HEPES buffer or HEPES buffer alone for 30 min at 37°C (post-stimulation) and the supernatant was collected for CGRP analysis. Capsaicin (50 nM) in HEPES (10 min at 37°C) was used as a positive control. CGRP levels (pg/ml) in the supernatants were determined with enzyme immune assay (EIA) kit in accordance with the manufacturer's instructions. The % change between pre and post-stimulation was calculated for each well.
3.3.2.2 Calcium imaging

After 24 and 48 h in culture, the cells were loaded with Fluo-3 (4.4 µM) for 30-40 min at room temperature (20–22 °C). The cells were washed with modified HEPES buffer (145 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM Glucose and 10 mM HEPES, pH 7.4 with NaOH) and then placed in the recording chamber and continuously perfused with bath solution (modified HEPES buffer) at a constant flow rate (1 ml/min). The calcium imaging was performed using a Nikon Diaphot inverted microscope with a diode laser 488 nm excitation and a 40X oil immersion objective. The change in emission (506 nm) i.e. intracellular calcium bound to Fluo-3 was recorded every 15-20 s using a PMT. Human ACPA (1 µg/ml) or control FT (1 µg/ml) was applied for 5 min to the same cells with a minimum of 10 min wash period between applications. At the end of each experiment 50 mM KCl was applied for 1 min as a control. All reagents were prepared from stock solutions and dissolved in modified HEPES buffer. Images were analyzed with ImageJ software. In each image, capturing in average 10 cells, all visible cells were chosen for analyses. Mean fluorescence intensity (F) for the region of interest (ROI), the cell bodies, was measured in each image. F₀ was calculated as the average mean intensity of the first 7 images in each series and the data presented as F/F₀.

3.3.2.3 Electrophysiological recordings

Whole cell patch-clamp recordings were performed at room temperature (20–22 °C) within 24 and 48 h of culturing using an Axo-Patch-200A amplifier filtered at 1 kHz and sampled at 4 kHz and analyzed Clampex 10.4.software. Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter) using a vertical puller. The resistance of the patch pipettes was 4–5 MΩ when filled with internal solution (120 mM K+-gluconate, 20 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 2 mM NaATP, pH 7.15 with Tris-base). Voltage-clamp recordings were conducted in small DRG neurons (15-25 µm in diameter) using the whole-cell configuration. Series resistance was not compensated. DRG neurons were continuously perfused with oxygenated bath solution (modified HEPES buffer) at a constant flow rate (1-1.5 ml/min). Human ACPA (1 µg/ml) was applied for 1 min and capsaicin (0.5 µM) was applied at the end of each recording for 10 s as a control (4 min wash period between applications). Cells having a resting membrane potential more negative than -40 mV were accepted. All reagents were prepared from stock solutions and dissolved in modified HEPES buffer and applied via an 8-channel pressure-controlled application system.

3.4 TISSUE ANALYSIS

3.4.1 Joint histology

Mice were deeply anesthetized with pentobarbital (50 µg/kg) and perfused intracardially with saline followed by 4% PFA. After perfusion, hind legs were post-fixed in 4% PFA for 48 h,
then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in a PBS (pH 7.4) at 4°C for 4-5 weeks, with changes of EDTA every 7 days. Tissues were then dehydrated in ethanol and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin and scored by blinded investigators as previously described (Bas et al., 2012) on a scale from 0-3, where 0 is normal and 3 is severe synovitis, bone erosion, and/or cartilage destruction.

3.4.2 Joint and bone immunohistochemistry

Following decalcification as above, each joint was cryoprotected in 30% sucrose at 4°C for at least 48 hours before being sectioned. To assess the binding of ACPA in different cell populations, either 30 µm-thick frozen sections of the bone/joint were obtained using a cryostat or mouse bone marrow cultures grown on glass slides, were used. Then, slides were dried at room temperature (RT) for 30 minutes, washed in 0.01M PBS three times for 10 minutes each, blocked with 3% normal goat serum (NGS) in PBS with 0.3% Triton-X 100 for 60 minutes and then incubated overnight with ACPA (5 µg/ml) with different primary antibodies made in 1% NGS and 0.1% Triton-X 100 in 0.01M PBS at RT. Blood vessels were labeled with an antibody against platelet endothelial cell adhesion molecule (rat anti-mouse PECAM, 1:500, BD PharMingen). Macrophages and osteoclasts were identified with an antibody against a single-chain glycoprotein of 110 kDa that is expressed predominantly on the lysosomal membrane of myeloid cells (rat anti-mouse CD68; 1:2000). Multinucleated CD68-positive stained and located within the mineralized bone-lining zone were considered to be osteoclasts. Primary afferent sensory nerve fibers were labeled with an antibody against calcitonin gene-related peptide (CGRP, polyclonal rabbit anti-rat CGRP; 1:5,000). After primary antibody incubation, slides were washed 3X10 minutes in PBS and incubated for 3 hours at RT with respective (488 and 594) Alexa-conjugated secondary antibodies (1:300). Sections were washed in 3X10 minutes in PBS and counterstained with DAPI (1:20,000) for 5 minutes. After this, preparations were then washed 3X10 minutes each in PBS, dehydrated through an alcohol gradient (70%, 80%, 90%, and 100%) for 2 minutes each wash, cleared in xylene for 2 minutes, and cover slipped with di-n-butylphthalate-polystyrene-xylene.

3.4.3 DRG and Spleen immunohistochemistry and immunocytochemistry

Mice were deeply anesthetized with pentobarbital (50 µg/kg) and perfused with saline followed by 4% paraformaldehyde (PFA). Following perfusion, the lumbar DRGs (L4-L6) and spleen were dissected, post-fixed (3 h for DRG, overnight for spleen) in 4% PFA, cryoprotected in 20% sucrose for two days and then frozen in OCT. The tissues were stored in -70°C until cryo-sectioned (12 µm) and mounted on glass slides. Cultured DRG primary cells were fixed in 4% PFA for 10 min 48 h after culturing and then stored in PBS at 4°C until stained. Non-specific binding in sections and cultured cells was blocked using 5% donkey serum in PBS. Tissues and cells were incubated with the primary antibody overnight at 4°C and the immunoreactivity was visualized using secondary donkey antibodies conjugated to Alexa 488 or Alexa 594 (1:300). Prolong Gold antifade with DAPI, was used for cover slipping. Antibodies against the following proteins were used: FcγRI, FcγIIb, FcγIII, and FcγRIV (2 µg/ml, gift from dr Mark Cragg, Uni. of Southampton. Antibodies
characterized in (Tutt et al., 2015)), PGP9.5 (1:500), CGRP (1:10 000, gift from Prof T. Hökfelt, Karolinska Institutet, Stockholm), TRPV1 (1:100), Iba1 (1:1000), GFAP (1:1000).

3.4.4 Confocal microscopy

Confocal images were acquired with a LSM710 confocal laser-scanning microscope operated with ZEN2012 software (Zeiss). Nuclear staining using DAPI; a fluorescent stain that binds strongly to A-T rich regions in DNA was visualized using an excitation beam of 405 nm and emissions were detected using a BA430-470 emission filter. Sequential acquisition mode was used to reduce bleed-through from fluorophores. Multipanel figures were assembled in Adobe Illustrator CS6 software.

3.4.5 Western blot

The presence of human IgG antibodies (ACPA, FT, and IgG from healthy controls) in mouse tissues and plasma was assessed by Western blotting. Joints (ankle), dorsal root ganglia, adipose tissue (subcutaneous white), skin (plantar hind paw), spleen, lung, skeletal muscle (quadriceps), heart, kidney, liver, spinal cord (L4-L6), brain and bone marrow (tibial) were homogenized with protein extraction buffer (0.5% Triton X-100, 50 mM Tris, 150 mM NaCl, 1mM EDTA and 1% SDS, pH 7.4) supplemented with proteases inhibitors (GE Healthcare). Supernatants from the homogenates as well as sera were mixed with LDS sample buffer containing DTT, and denatured at 70°C for 10 minutes. Total proteins from the tissue homogenates and plasma (30 µg per well) were loaded onto NuPAGE® Bis-Tris 4-12% gels and run in MES-SDS antioxidant-containing running buffer at 200 V for 50 min. Proteins were transferred to a nitrocellulose membrane at 30V and blocked with 5% non-fat dry milk prepared in TBS containing 0.1% Tween 20 for 1 hour at room temperature. For the immunoblotting, membranes were incubated with the secondary antibody rabbit anti-human IgG HRP (1:10 000) for 1 hour at room temperature. The membranes were developed using the SuperSignal® West Pico chemiluminescent substrate, according to manufacturer’s instructions.

3.4.6 Gene expression analysis

3.4.6.1 Quantitative real-time polymerase chain reaction (PCR)

Ankle joints and plantar paw skin of the hind legs were processed for gene expression analysis. Muscle and tendons were removed from ankle joints, which were then snap frozen and pulverized. Tissues were sonicated in TRIzol and RNA was extracted according to manufacturers protocol. After complementary DNA (cDNA) synthesis, quantitative real-time PCR was performed using hydrolysis probes to determine the relative messenger RNA (mRNA) levels. Primers for various genes (Table 3), including reference gene Hprt1, were used to determine threshold cycle values to calculate the number of cell equivalents in each sample with the standard curve method. Data is normalized to Hprt1 values and expressed as relative expression units (REU).
Table 3. Primers used for real-time PCR

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<tbody>
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<td>Ccl2 (Mcp-1)</td>
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<tr>
<td>Tpsb2 (Mcp6)</td>
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</tr>
</tbody>
</table>

3.4.6.2 Microarray expression analysis

Lumbar DRGs (L4-6) were dissected and total RNA was extracted and purified using an RNeasy mini kit according to manufacturers instructions. RNA concentration was measured using an ND-1000 spectrophotometer and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system. 250 ng of total RNA from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit and AffymetrixGeneChip® WT Terminal Labeling and Hybridization User Manual. GeneChip® ST Arrays were hybridized for 16 h in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G. The raw data was normalized in the free software Expression Console provided by Affymetrix using the robust multi-array average (RMA) (method first suggested by (“Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection,” 2001)), followed by extraction of the expression levels of the genes of interest (Fcgr1, Fcgr2b, Fcgr3 and Fcgr4). For human comparison, the gene expression levels (GeneChip® Human Genome U133 Plus 2.0) from eight donor human dorsal root ganglia, of the corresponding human genes (FCGR1C, FCGR2A, FCGR2B, FCGR2C, FCGR3A, FCGR3B and reference genes TRPV3, TRPV4, HTR3C), were extracted from the ArrayExpress publically available gene expression set (Roth et al., 2006).

3.4.6.3 Single-molecule fluorescence RNA in situ hybridization (smFISH)

smFISH was carried out as previously described (Zeisel et al., 2015) with some modifications. DRG from mice were collected and frozen in OCT. After cryosectioning, the sections were post-fixed in 4% PFA, and stored in -80°C until use. For hybridization, the sections were first permeabilized for 10 min with methanol in -20°C and incubated with 250 nM fluorescent labeled probes for 4h at 37°C and counterstained with DAPI. The sections were mounted with Pro-long Gold and image stacks (0.3 µm distance) were acquired using a customized automated scanning microscope controlled by µManager. Images were analyzed using a custom Python script. After background removal, a Laplacian-of-Gaussian was used
to enhance the RNA dots that were identified by computing the extended maxima-transform of the flattened image.

3.5 STATISTICS

For comparing changes over time, repeated measures two-way analysis of variance (ANOVA) was used followed by a Bonferroni post-hoc test. For differences in three groups or more, a one-way ANOVA was used, followed by a Bonferroni post-hoc test. For differences in two groups, Students t-test was used. Arthritis and histological scores were compared using the Kruskal-Wallis test followed by Dunn’s multiple comparison post hoc test. All tests were performed using GraphPad Prism 6 software. P values less than 0.05 were considered significant. No statistical method was used to pre determine sample sizes.
4 RESULTS

The work in this thesis is divided into two main projects involving pain and autoantibodies: Paper I and II investigating effects of anti-citrulline immunity, and Paper III the role of anti-collagen antibodies.

4.1 THE ROLE OF ANTI-CITRULLINATED PROTEIN ANTIBODIES

ACPA has been thoroughly studied in regard to involvement in arthritis, but there is little evidence for a direct role in inflammatory pathogenesis. The interest to study ACPA from a sensory perspective originated because of certain features of the antibodies: they are commonly present in patients years before onset of RA, a period associated often with arthralgia, and the more recent discovery that they are linked to increased osteoclast (OC) activity and subsequent bone erosion.

4.1.1 ACPA induce pain-like behavior in mice

We started by comparing the effect of ACPA\(^+\) and ACPA\(^+\) IgG from RA patients on mechanical sensitivity of mice. Only antibodies from ACPA\(^+\) RA patients induced pain-like behavior in the mice. We then separated the IgG pool into ACPA and non-ACPA (FT), and determined that only the ACPA pool induced pain-like behavior. This included increased mechanical and thermal sensitivity, as well as reduction in locomotion. Importantly, there was no visual sign of inflammation in the mice, and there was no display of inflammation-related sickness behavior like piloerection, weight loss or reduced feeding. Thus, we concluded that the observed effects were a consequence of the pro-nociceptive effect of ACPA (Fig. 9). Additionally, we also tested murinized monoclonal ACPA that were cloned from single B cells from RA patients and tested for CCP reactivity. These antibodies were also able to induce pain-like behavior in the mice, meaning that the observed effect was not associated with a reaction against human protein.
Figure 9. Behavioral effects after injection of human antibodies. Mechanical sensitivity in mice after injection of IgG from healthy donors, IgG from patients with ACPA- or ACPA+ RA (A), purified ACPA or non-ACPA (FT)(B). Arthritis scores (0-60) in injected mice (C). Thermal sensitivity and changes in total movement in mice injected with ACPA or FT (D-F).

4.1.2 ACPA accumulate in joints and bone marrow but does not directly increase neuronal excitability or induce signs of inflammation

To investigate how ACPA induce pain-like behavior, we first wanted to determine where the antibodies go in the mouse. This was done by perfusing the mice with saline to eliminate the blood and then performing Western blots on the tissues, to detect human IgG. We found that IgG from healthy controls and FT distributed similarly in most tissues, while ACPA had a more restricted localization to joint, tibial bone marrow, and skin. Importantly, there were no antibodies in the CNS, indicating that induction of nociception is from peripheral locations. Many ACPA are produced by B cells in the arthritic synovia thus affinity maturation is driven by local antigens, but their preference to that location in naïve mice is an interesting observation.

Next we wanted to examine the effects of the antibodies. An obvious possibility is that ACPA directly activate sensory neurons, causing nociceptive signaling. To test this we applied the antibodies to cultured DRG neurons, but ACPA did not induce intracellular Ca\(^{2+}\) or changes in membrane current, suggesting no direct effect on neuronal activity. Even though we could not visually detect inflammation, there is a possibility that low-grade inflammation could cause pain-like behavior. So we analyzed histological sections of tibial bone and joint, gene expression of inflammatory markers, and MMP activity, but there were no signs of cell infiltration, increased expression or activity of factor associated with inflammation. Noteworthy, however, Cxcl1 and Cxcl2 mRNA levels were elevated in ankle joint from ACPA, but not FT or saline-injected mice. These factors were not elevated in skin from the
plantar surface of the hind paw, which is important since we detected ACPA there and that is the region mechanical and thermal sensitivity is measured in the mice.

### 4.1.3 ACPA bind osteoclasts and induce CXCL1/IL-8 release in mice and men

In parallel work, we were investigating how ACPA interact with human OCs. It became clear from cell cultures that both polyclonal and monoclonal ACPA bound to OCs, and its precursors, causing both proliferation and increased calcium phosphate resorption. This process was dependent on PAD activity, since Cl-amidine, a pan-PAD inhibitor, was able to inhibit both the ACPA-induced proliferation and resorption. To investigate potential mediators responsible for the effect of ACPA, we analyzed the supernatant of the cultures for a common set of cytokines, including IL-6, TNF, and IL-10. Interestingly, all measured cytokines had low constant levels, except IL-8, which showed a significant increase with ACPA treatment. Adding exogenous IL-8 increased osteoclastogenesis and blockade of extracellular IL-8 with a neutralizing antibody inhibited the ACPA-induced OC formation, implicating IL-8 as a key mediator in the process.

Importantly, using bone marrow cultures from mice we were able to show a similar pattern, ACPA bind OCs and induce release of CXCL1, which is the murine functional analogue of human IL-8. To connect the localization data with the in vitro effects, we wanted to determine the cellular targets of ACPA using immunohistochemical labeling of section from joint and bone (Fig. 10). This revealed that ACPA bind CD68+ cells with multinucleated morphology, proximal to mineralized bone, most likely OCs, as well as its precursors in the bone marrow. Interestingly, some ACPA+ cells were located in close proximity to CGRP+ sensory fibers in the bone marrow, showing a histological link.

![Fig 10. Binding of ACPA in bone marrow. Co-localization of ACPA with marker for macrophage/osteoclasts (CD68) in subchondral bone (A) and marker for sensory nerve fiber (CGRP) in tibial bone marrow (B). ACPA and CD68 binding in cultured mouse osteoclasts (C). Scale bar is 25 µm.](image_url)
4.1.4 Pain-like behavior and bone erosion in mice is dependent on CXCL1/2

We had several lines of evidence pointing towards OC activation and release of CXCL1 explaining the ACPA-induced pain-like behavior, but we needed to show the connection in vivo. First we investigated the nociceptive effect of CXCL1/2, by injecting these factors into the ankle joint. This caused onset of robust mechanical hypersensitivity in the ipsilateral paw, confirming the nociceptive potential of the factors. To further examine the link to ACPA, we treated mice that had ACPA-induced thermal and mechanical hypersensitivity with the CXCR1/2 receptor antagonist reparixin. Six consecutive days of treatment partially reversed the mechanical and thermal hypersensitivity. Additionally, the tibia of the mice were analyzed using micro-CT revealing that ACPA induced erosions in the trabecular bone, which was inhibited by the reparixin treatment (Fig. 11).

Figure 11. Effect of ACPA and treatment with reparixin on tibial bone parameters. Representative 2D micro-CT images of the tibial metaphysis of control mice (A) and mice that were injected with ACPA in the absence (B) or presence of reparixin (C). Graphs showing quantitative evaluation of the trabecular bone mineral density (BMD, D), trabecular number (E), bone volume fraction (bone volume/tissue volume, F) and the cortical tissue mineral density (TMD, G).
To summarize (Fig. 12), we propose a mechanism where ACPA bind to citrullinated antigens on osteoclasts, causing activation and release of CXCL1/IL-8. This causes increased proliferation and bone resorption by the osteoclasts. The CXCL1/IL8 activates and sensitizes local sensory neurons in the bone and joint, producing pain-like behavior manifested as reduction in the utilization of the joints, i.e. reduction in locomotion. The increased sensitivity also spreads to adjacent tissues, such as the plantar paw, reducing cutaneous thermal and mechanical thresholds for activation of the sensory fibers. This secondary hypersensitivity could be mediated either by a peripheral effect, where activity in a fiber can affect other fibers in the same nerve bundle (Sheth et al., 2002), or centrally by nociceptive circuit amplification and facilitation (Basbaum et al., 2009; Campbell et al., 1988).

4.2 THE ROLE OF ANTI-COLLAGEN ANTIBODIES IN INDUCTION OF NOCICEPTION

In our previous work with the CAIA model, we found that pain-like behavior is apparent several days before the onset of inflammation. We predicted that the early phase of the model would give us important information about the mechanisms that drive arthralgia in the “pre-RA” phase.

4.2.1 Induction of pain-like behavior is not associated with inflammation

Our initial experiments aimed at determining if there was a low-grade inflammation, not detectable by visual inspection, which could explain the pain-like behavior. Analysis of histological sections from the mice 5 days after injection of antibodies showed little signs of cell infiltration, bone erosion or cartilage destruction. We then decided to analyze the gene expression in the ankle joints, to see if there was elevation of any factors associated with arthritis pathology or pain signaling, like Tnf, Cox2, and MMPs. None of the factors we analyzed were elevated 5 days after injection of antibodies. We also analyzed the activity of MMPs using MMPsense, which revealed no increased activity of the enzymes. These results suggest that other factors than those involved in innate inflammatory and bone remodeling drive anti-CII mAb induced mechanical hypersensitivity prior to onset of joint inflammation.
4.2.2 Pain-like behavior was apparent as early as two days after injection of antibodies

To determine the temporal profile of the pain-like behavior, we examined the thresholds for response to mechanical stimulation every day during the 5 first days after injection of anti-CII antibodies (Fig. 13). By day 2 there was already a significant reduction in tactile thresholds compared to control mice. We decided to monitor mouse locomotion, which is a non-evoked measure that can indicate spontaneous pain in rodents, around that time point. The measurement takes place during the whole night, which is their active period. Antibody-injected mice showed reduction in total movement, ambulatory movement, as well as in rearing.

Figure 13. Anti-CII antibody-induced behavioral changes.
Anti-CII cocktail was injected and mechanical sensitivity assessed (A), visual signs of inflammation expressed as arthritis score 0-60 (B), and arthritis incidence (C). Locomotor activity was monitored during the third night (12 h) after injection (D).

4.2.3 Antibody epitope recognition, but not pathogenicity, is important in early antibody induced pain-like behavior

The cocktail of antibodies (M2139, CIIC1, UL1, and CIIC2) used to induce CAIA are directed against four conserved epitopes present on both human and mouse CII protein. It has been demonstrated that these antibodies are associated with varying degree of pathogenicity when injected alone. We wanted to test if this factor is linked to the ability to induce pain-like behavior, so we injected the antibodies separately. Interestingly, each antibody induced a similar degree of hypersensitivity, this was comparable to results following injection of the mixed cocktail. Isotype control antibodies did not induce any changes. Injection of only M2139 reduced locomotion in the mice, as well as causing long-term mechanical hypersensitivity at doses that did not induce joint inflammation for at least 21 days.

The antibody ACC4 binds the citrullinated C1 epitope on CII but is not able to induce inflammation on its own, making it an interesting comparison to the previous anti-CII
antibodies. Surprisingly, the ACC4 induced similar mechanical hypersensitivity and reduction in locomotion as the antibodies binding to native CII. Taken together, this suggests that although epitope recognition is critical, antibody-induced pain-like behavior is not coupled to the pathogenicity of the antibodies.

4.2.4 Complement activation or changes in cartilage structure does not contribute to early pain-like behavior.

One of the primary effector functions for antibodies is activation of the complement cascade through the classical pathway. This leads to release of complement component 5a (C5a), which is able to directly activate nociceptors. We thought this could be a potential mechanism of induction of pain-like behavior. To test this, we used pharmacological inhibition of the C5 receptor using a peptide antagonist and performed experiments using congenic mice deficient in the C5 protein. Neither of these experiments prevented changes in locomotion or mechanical hypersensitivity, thus not supporting a major role for complement activation in antibody-induced nociception.

The anti-CII antibodies have been shown to destabilize the cartilage structure in vitro and cause an early loss of cartilage proteoglycans in vivo, demonstrating a direct effect on cartilage. To determine if induction of pain-like behavior is linked to these processes we used ACB mice with or without collagen typ IX (CIX−/−) deficiency. The ACB mouse is an IgG heavy chain knock-in mouse that spontaneously produce anti-CII antibodies that bind to the C1 epitope. Collagen type IX stabilizes the cartilage structure and absence of CIX leads to an increased cartilage porosity, enabling deeper penetration of the antibodies. The ACB and the ACB CIX−/− mice did not differ in tactile threshold baselines, and when injected with anti-CII antibodies, they both developed both evoked and spontaneous pain-like behavior to a similar degree. This indicates that stability and integrity of cartilage did not influence early pain-like behavior.

The CIIF4 antibody binds CII but does not induce any changes in the cartilage structure and protects antibody-mediated cartilage damage both in vivo and in vitro. In fact, it prevents development of arthritis when injected together the anti-CII cocktail. Interestingly, the injection of CIIF4 antibody in mice produced robust mechanical hypersensitivity of the same degree as anti-CII mAbs. Taken together, these data further support our findings that change in cartilage structure and pathogenicity of the antibody are not critical factors for induction of antibody-mediated nociception.

4.2.5 CII immune complex has a direct effect on cultured DRG neurons

Based on the finding described above, we formulated the hypothesis that anti-CII antibodies may have a direct effect on peripheral neurons, through mechanisms that are uncoupled from the inflammatory process. In order to test this hypothesis, primary DRG neuronal cell cultures were prepared and stimulated with immune complex (IC) made from anti-CII antibodies and
the protein CII. The immune complex, but not anti-CII antibodies, CII alone, or isotype controls, induced release of the peptidergic neurotransmitter CGRP. Expression of CGRP is common in nociceptors innervating the joint and bone.

In order to further investigate the potential role of CII-IC as a direct activator of nociceptive sensory afferents, we used two other functional assays commonly used for assessment of neuronal activity. We first investigated the effect of CII-IC on intracellular Ca\(^{2+}\) levels (Fig. 14). Application of CII-IC caused a Ca\(^{2+}\) response in 22% of the neurons (responding to KCl). To simulate more physiological conditions, we also pre-incubated the cultures with isotype control antibodies before application of the IC, giving a similar response rate of 21%.

Next we performed electrophysiological recordings in small diameter neurons, which are mostly C-type primary afferents and more likely to mediate nociceptive signals than the large diameter A-type afferents. We focused on a subpopulation of small diameter nociceptive neurons that express TRPV1, and we used the TRPV1 agonist capsaicin for verification at the end of each experiment. Of the cells recorded, 48% gave inward current responses to capsaicin and of those, CII-IC evoked inward current in 42%.

![Figure 14. The effect of CII-IC on cultured DRG neurons. Representative traces are showing increased intracellular Ca\(^{2+}\) in response to CII-IC but not isotype control antibody IgG2b.](image)

### 4.2.6 FcγRs are present on sensory neurons

Our data suggest a direct action of CII-IC on sensory neurons, thus implicating a role of receptors for IgG: FcγR. We started by analyzing gene expression data from DRG mRNA extracts, which revealed that all transcripts were present (Fcgr1, Fcgr2b, Fcgr3, and Fcgr4), with the highest expression of Fcgr2b and Fcgr3. This was confirmed with quantitative real time PCR, which presented with a similar expression pattern. We also analyzed publically available gene expression data from human DRG samples, which showed mRNA of all FCGRs with the highest relative expression of FCGR2A and 2C. The presence of FcγR protein in mouse DRG was determined by proteomic analysis using high performance nanoLC-MS/MS. By doing so, we detected two unique peptides originating from FcγRIIb, along with two other non-unique peptides shared between FcγRIIb/III. Peptides from FcγRI or FcγRIV were not detected. To determine the cellular localization of FcγRIIb, single
molecular fluorescence in situ hybridization (smFISH) was used, showing that \textit{Fcgr2b} transcripts were restricted to a subset of neurons in the DRG. Additionally, by using immunohistochemistry, FcyRIIb was located to neurons in the DRG, while FcyRI and FcyRIII were restricted to non-neuronal cells. Presence of FcyRIIV was not detectable. Co-localization studies showed that FcyRIIb is expressed in small and medium sized neurons, including TRPV1$^+$, CGRP$^+$, and non-peptidergic IB4$^+$ neurons (Fig. 15).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure15.png}
\caption{Figure 15. Presence of FcyRs in DRG. Relative expression of mRNA in mouse for \textit{Fcgr1-4} using microarray (A) and qPCR (B). Relative expression of mRNA in human DRG for \textit{FCGR1-3B} using microarray (C). Representative result from smFISH for \textit{Fcgr2b} (green) and DAPI (blue) in mouse DRG (D). Immunoreactivity for FcyRI (E), FcyRIIb (F), FcyRIII (G), and FcyRIV (H) in mouse DRG.}
\end{figure}

\subsection{4.2.7 Antibody – FcyR interaction is necessary for nociceptive effect \textit{in vivo}}

In order to investigate if FcyR binding the Fc part of the antibody is required for nociceptive effects, we removed the Fc from the anti-CII antibodies and injected the remaining Fab-fragments and measured mechanical sensitivity and locomotor activity. Mice injected with anti-CII mAb Fab did not develop any signs of evoked or spontaneous pain-like behavior and did not differ behaviorally from control mice injected with saline. Antibodies with an
endoglycosidase (EndoS) hydrolyzed glycan on the heavy chain (γ-chain) at asparagine 297 have a diminished affinity for FcγR interaction. To test the functional importance for Fc mediated FcγR activation in antibody-induced nociception, EndoS treated anti-CII antibody was injected to a group of mice. EndoS-treated antibodies did not induce mechanical hypersensitivity or reduction in locomotor activity compared to control mice. Additionally, anti-CII mAbs were injected in mice that lack the common γ-chain, thus without functional FcγRI, FcγRIII, and FcγRIV, and compared with wild type. The FcRγ-/- mice were resistant to antibody-induced mechanical hypersensitivity. These results indicate that the binding of Fab to CII is not sufficient to activate nociceptors, that glycosylated Fc and presence of activating FcγRs are required. Hence the antibody-FcR interaction is necessary in vivo for antibody-induced pain-like behavior.

To summarize, we propose a mechanism that is dependent on antibody interaction with FcγRs for induction of pain-like behavior. Anti-CII antibodies bind the cartilage in the joint, forming IC that can activate local FcγRs. The activation of FcγRs on local immune cells causes release of factors that prime sensory neurons in the joint area. These neurons can be directly activated by the ICs through the expression of FcγRIIb, present on both peptidergic and non-peptidergic neuronal populations. Disruption of the antibody – FcγR crosslinking inhibits the pain-like behavior.
5 DISCUSSION AND CONCLUDING REMARKS

This thesis focuses on mechanisms of pain in autoimmunity and how antibodies interact with the sensory system. The nervous and immune systems have traditionally been regarded as two separate entities with separate functions and structures, and thus are studied separately. Information in the nervous system is relayed quickly by electrical signals through relatively static nerve fibers, while the immune system is inherently mobile and dispersed, depending on the movement of cells through tissues and into the circulation. Both systems are critical for an organism responding to the danger imposed on tissue homeostasis, and both are highly adapted to sense both internal and external environments. However, emerging evidence points towards extensive crosstalk between the two systems, acting in coordinated ways to promote an effective defense for the organism. The systems communicate through a common language of cell-surface receptors and released proteins and peptides. In fact, as a greater number of mediators that are common to the immune and nervous systems are discovered, terms such as “cytokine” and “neuropeptide” becomes misleading.

Unfortunately, being a vital component for the health of organisms, neuro-immune crosstalk also contributes to disease states, including autoimmunity. A better understanding of the coordinated interaction of peripheral neurons with immune cells might advance therapeutic approaches, both in suppressing the immune system and the mechanisms contributing to chronic pain.

In Paper I and II we investigated the effect of anti-citrulline immunity and found that ACPA induced osteoclast activity, causing bone erosion and pain-like behavior in mice (Fig. 16). It introduces a new perspective on the role of antibodies against citrullinated proteins, of which little is known. As with most research, the results lead to more questions than answers. A fundamental question that needs to be addressed is which epitope(s) are targeted and what mechanism triggers the CXCL1/IL-8 release? The CCP assays used for diagnosis use a generic synthetic peptide that is not present in vivo. Several citrullinated epitopes on proteins are targeted by ACPA, including CEP-1, vimentin, and fibrinogen, but most monoclonal antibodies studied so far show remarkable cross-reactivity (Amara et al., 2013). Additionally, the citrullination status of individuals changes during the disease course, inflammation in a joint is associated with increased citrullination (Romero et al., 2013), which is quite distinct from a naïve state. We show that citrullinated antigens are a vital part of osteoclast formation, and the antibodies bind those structures. It is possible that other cell systems also use citrullination during normal physiology, which could potentially be targeted by ACPA. Since we find that some monoclonal ACPA do not induce pain-like behavior, it is important to find which epitopes are involved to be able to predict anything about pain in patients, and potentially device serological tests based on this.

The ACPA we have used in these studies, both monoclonal and polyclonal pool, comes from diagnosed patients at the rheumatology clinic, i.e. persons that had autoantibodies for some
time. It would certainly be informative to harvest antibodies and cells from individuals longitudinally, to establish the nociceptive and erosive effects of ACPA during the maturation of the disease. Especially around the time when there is a rapid change in the anti-citrulline immunity: sharp increases in antibody levels and number of specificities, as well as epitope spreading (Sokolove et al., 2012). In practice, this would be hard to perform but would give interesting information about the time point when the autoantibodies switch from benign to symptomatic.

With a more detailed knowledge about the effects of anti-citrulline immunity, the term ACPA-positive could be classified as a disease state or syndrome. This would warrant earlier clinical treatment and hopefully inhibit progression into RA.

In Paper III we investigate the role of anti-CII antibodies in initiation of nociceptive signaling, and find that anti-CII in immune complex formation directly activate sensory neurons through FcγRs, likely causing pain-like behavior in mice (Fig. 16). The anti-CII antibodies are quite distinct in some ways from ACPA, in that the precise epitope binding is well characterized and the effect this has on the collagen protein. The similarity is the relevance to RA, since anti-CII antibodies are present in some RA patients, targeting the same conserved epitopes. Additionally, these antibodies are also able to induce pain-like behavior without signs of inflammation, indicating a more complex effect than just activating immune cells.

After injection, the anti-CII antibodies rapidly bind cartilage in the joints (Nandakumar et al., 2003). The ability of the anti-CII antibodies to disrupt the cartilage structure in combination with availability of soluble CII in the normal synovial fluid (Lohmander et al., 2003), provides opportunity to form soluble ICs that can activate local FcγRs, in particular on the sensory neurons innervating the synovial tissue and bone structures. The pain behavior is also dependent on activation on local FcγR-expressing cells, most likely resident macrophages or fibroblast-like synoviocytes, to release factors that activate the neurons. Since mice lacking activating FcγRs do not display pain-like behavior, they may be important in activating these local immune cells to recruit silent nociceptors (Schaible et al., 2009). However, this activation of local cells is not sufficient for a detectable inflammatory response.

We show for the first time the presence of FcγRIIb on sensory neurons. Previous in vitro effects by ICs on cultured DRG neurons has been attributed to FcγRI (Andoh and Kuraishi, 2004; Qu et al., 2011). In some cases this could be explained by species differences, but a more likely factor is the availability of specific tools. Often in these reports, the method for determining cellular localization is based on immunoreactivity, which can be deceptive. We use several method to establish confidence in receptor expression. The notion that FcγRIIb is able to mediate an activating effect on neurons is quite interesting, since it is classically thought of as an inhibitory receptor on immune cells, either inhibiting the activating receptors or mediating endocytosis (Nimmerjahn and Ravetch, 2006). Early reports of direct effects of ICs on motor neurons show internalization in addition to activation (Mohamed et al., 2002).
The intracellular mechanisms of antibody-FcγRIIb crosslinking is certainly important to further explore.

The interaction between sensory neurons and the immune system during autoimmunity may have consequences for the progression of the disease as well. Activated sensory neurons release neuropeptides from their peripheral terminal, and immune cells express receptors for almost all of these known peptides (Talbot et al., 2016). For example, the neuropeptide CGRP has complicated modulatory effects on immune cells, important in antimicrobial defense (Augustyniak et al., 2012). Innervation and neuronal activity has been shown to regulate inflammation, where denervation protects patients and rodents from development of arthritis (Courtright and Kuzell, 1965; Stangenberg et al., 2014). This suggests that the sensory neurons have a vital role in regulating the local environment in the joint and cellular activity. Additionally, many immune cells produce and secrete NGF, an important growth factor for neurons that can trigger nociceptive neurons and remodeling of peripheral innervation (McMahon et al., 2015; Shepherd et al., 2005). This can create a neuro-immune amplification loop, in which immune cells secrete cytokines that activate neurons, and neurons secrete peptides that activate immune cells. During chronic autoimmune disease, this creates a primed state so flares can easily be triggered, due to disproportionate responses of the immune cells and pain fibers (Talbot et al., 2016). Thus, for patients, targeting both the overactive immune system and sensory system could potentially lead to a complete resolution of symptoms. Here we provide evidence that autoantibodies can be an important part of that loop, and a possible target for intervention against chronic pain.

![Figure 16. Summary of proposed pain-inducing mechanisms. 1) ACPA binds citrullinated epitopes on osteoclasts, causing release of CXCL1/IL-8 that can activate and sensitize sensory neurons, through the receptor CXCR2. 2) Antibodies in IC-formation can directly activate sensory neurons through FcγRs, causing pain-like behavior.](image)

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