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MOLECULAR STUDIES ON THE AUTOANTIBODY-MEDIATED BONE DESTRUCTION IN RHEUMATOID ARTHRITIS

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Molecular Studies on the Autoantibody-Mediated Bone Destruction in Rheumatoid Arthritis
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To my beloved family

ABSTRACT

Autoantibody-positive rheumatoid arthritis (RA), also called seropositive RA, is characterised by the presence of anti citrullinated proteins antibodies (ACPA) that can be detected in blood several years before disease onset. Joint inflammation, pain and bone destruction are major features of the disease. Classically bone destruction and pain have been considered to be late events in the disease development, resulting from long lasting and uncontrolled inflammation. However, both bone loss and pain have been reported in both seropositive individuals not yet having the disease and those seropositive individuals just being diagnosed with seropositive RA. Taken together these findings suggest that other factors than uncontrolled joint inflammation might drive the process of bone loss and pain in RA. We hypothesized that antibodies themselves and specifically ACPA might play a direct role in mediating bone loss and pain and aimed to investigate the role of ACPAs in bone metabolism and pain-like behaviour in mice.

To investigate bone metabolism, we focused on studies of osteoclasts (OC), cells responsible for bone loss in vitro (by assessing formation and maturation of OC in cell cultures and estimating their capacity to degrade bone matrix in vitro) and in vivo (by micro-CT analysis of the bone density). We have demonstrated that polyclonal and monoclonal ACPAs isolated from the blood and synovial fluid of RA patients enhance the number of OC and of their bone resorptive capacity in vitro. This effect was accompanied by a significant increase of IL-8 levels in OC supernatants and abolished by neutralizing anti IL-8 antibodies. Further, ACPA injected in mice were shown to bind to CD68-positive OC precursors in bone marrow in the vicinity of the joints and to promote trabecular bone loss, which was also reversed by blocking the mice homologues of IL-8, CXCL1/2 by using reparixin. In parallel to bone destruction ACPA also induced pain-like behaviour in mice, that similar to bone loss was also abolished by CXCL1/2 blocking. Taken together these findings suggest that ACPA promote bone loss by inducing IL-8 that in turn can further amplify the bone loss process and induce pain-behaviour.

As ACPA, but no other immunoglobulins (non-ACPA immunoglobulins from RA patients, non-ACPA Ig from healthy individuals) were able to promote bone loss, we investigated the role of citrullination in ACPA-mediated osteoclastogenesis. We demonstrated that citrullination by peptidyl arginine deiminases (PAD) enzymes is essential for the physiological development and maturation of OCs but no other cells (such as synovial fibroblasts). This finding might explain the ACPA preference for OCs. Further we showed that ACPA bind to targets expressed on the surface of OCs. Blocking the citrullination

machinery by PAD enzyme inhibitors significantly abrogates ACPA binding to OCs and ACPA-mediated osteoclastogenesis.

OCs could develop from different cell precursors and inflammatory conditions, such as joint inflammation in RA, promote the transdifferentitation of immature DC (iDC) into OCs. In order to investigate if ACPA might also play a role in this proces, we analyzed the capacity of polyclonal and monoclonal ACPA to promote the in vitro transdifferentation of iDC to OC. We showed that despite a clearly distinct protein profile as compared to classical macrophage OC precursors, iDC are able to develop into remarkably similar OCs. Plasticity towards OC differentiation correlated with PAD activity and protein citrullination expression levels in iDC cultures. Citrullinated actin and vimentin were present in iDCs and iDC-derived OCs and both proteins were deposited on the cell surface, co-localising with ACPAs binding to the cells. ACPAs enhanced OC differentiation from both monocyte-derived iDCs and from circulating CD1c+ DCs. Blocking either PAD activity or ACPA-induced IL-8 secretion completely abolished the stimulatory effects of citrulline-targeting antibodies on DC-OC transdifferentiation. We further explored the mechanisms involved in the plasticity of iDC and their capacity to develop into OCs showing that cell culture densities and lactate concentrations are essential mediators. DCs originating from dense cultures developed in the presence of high lactic acid doses, have high PAD activity and increased efficiency to convert into OC and erode bone. In contrast, DCs from sparse cultures have low PAD activity with decreased OC potential.

In conclusion, the current thesis describes novel mechanisms by which RA-associated antibodies target OC to induce bone loss and pain. Our studies provide insights into the mechanisms by which systemic autoimmunity might target the joints and suggest potential novel ways to prevent this.

LIST OF SCIENTIFIC PAPERS

I. **Krishnamurthy A**, Joshua V, Haj Hensvold A, Jin T, Sun M, Vivar N, Ytterberg AJ, Engström M, Fernandes-Cerqueira C, Amara K, Magnusson M, Wigerblad G, Kato J, Jimenez-Andrade JM, Tyson K, Rapecki S, Lundberg K, Catrina SB, Jakobsson PJ, Svensson CI, Malmström V, Klareskog L, Wähämaa H, Catrina AI.
Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss.
Annals of the Rheumatic Diseases, (2016) –208093

II. Wigerblad G, Bas, DB, Fernandes-Cerqueira C, **Krishnamurthy A**, Nandakumar KS, Rogoz K, Kato J, Sandor K, Su J, Jimenez-Andrade JM, Finn A, Bersellini Farinotti A, Amara K, Lundberg K, Holmdahl R, Jakobsson PJ, Malmström V, Catrina AI, Klareskog L, Svensson CI.
Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism.
Annals of the Rheumatic Diseases, (2016) –208094

III. **Krishnamurthy A**, Ytterberg AJ, Sun M, Steen J, Joshua V, Tarasova NK, Malmström V, Wähämaa H, Réthi B and Catrina AI.
Citrullination controls dendritic cell transdifferation into osteoclasts and generates target for RA-associated autoantibodies
Manuscript.

IV. Nasi A[*], Fekete T[*], **Krishnamurthy A**[*], Snowden S, Rajnavölgyi E, Catrina AI, Wheelock CE, Vivar N, Rethi B.
Dendritic cell reprogramming by endogenously produced lactic acid. *Journal of Immunology,* (2013) - 3090-9
* Contributed Equally

PUBLICATIONS NOT INCLUDED IN THE THESIS

V. Neregård P, **Krishnamurthy A**, Revu S, Engström M, af Klint E, Catrina AI.
Etanercept decreases synovial expression of tumour necrosis factor-α and lymphotoxin-α in rheumatoid arthritis.
Scandinavian Journal of Rheumatology, (2014)- 834964

VI. Grönwall C, Amara K, Hardt U, **Krishnamurthy A**, Steen J, Engström M, Sun M, Ytterberg AJ, Zubarev RA, Scheel-Toellner D, Greenberg JD, Klareskog L, Catrina AI, Malmström V, Silverman GJ.
Autoreactivity to malondialdehyde-modifications in rheumatoid arthritis is linked to disease activity and synovial pathogenesis.
Journal of Autoimmunity, (2017)-30283-4

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
Arg	Arginine
APF	Antiperinuclear factor
BAL	Brohchoalveolar lavage
BMD	Bone mineral density
CCP	Cyclic citrullinated peptide
CIA	Collagen induced arthritis
Cit	Citrullinated
CD	Cluster of differentiation
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CTLA4	Cytotoxic T lymphocyte-associated protein 4
CXCL	Chemokine CXC motif ligand
CXCR	CXC chemokine receptors
DC	Dendritic cell
EULAR	European League Against Rheumatism
FLS	Fibroblasts like synoviocytes
FT	Flow through
G-MCSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leucocyte antigen
HRCT	High resolution computed tomography
FcγR	Fc gamma receptor
Fab	Fragment binding antigen
IC	Immune Complex
Ig	Immunoglobulin

IL	Interleukin
i.v.	Intravenous
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
OC	Osteoclast
MΦ	Macrophage
MCP	Metacarpophalangeal
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MTP	Metatarsophalangeal
PAD	Peptidyl arginine deiminase
PADI4	Peptidyl arginine deiminase 4 gene
PIP	Proximal Interphalangeal
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid Arthritis
RANKL	Receptor activator of nuclear factor kappa –B ligand
RF	Rheumatoid Factor
s.c.	Subcutaneous
SE	Shared epitope
SF	Synovial fluid
TNF	Tumour necrosis factor
TMD	Tissue mineral density
TRAF	TNF receptor-associated factor
TRAP	Tartrate-resistant acid phosphatase

1 INTRODUCTION

1.1 Rheumatoid arthritis

1.1.1 Definition and clinical picture

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by joint destruction and pain that might lead to longstanding physical disability affecting the quality of life in the absence of appropriate treatments (1, 2). RA has an annual incidence of 0.2% in males and 0.4% in females with a prevalence of around approximately 1% in the world population (1, 3). Pain and swelling of the small finger joints (metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints), in wrists and forefeet, mainly the metatarsophalangeal joints (MTP) are most common symptoms (4). If left untreated the disease leads to destruction of cartilage and bone. The clinical picture of the disease varies between individuals, with some presenting a more aggressive bone and cartilage destruction, while others have no erosion (5). The treatment today consist in often long life use of disease-modifying anti-rheumatic drugs (DMARDs), both synthetic DMARDs and biological DMARDs, a collection of heterogeneous treatment agents that reduces the joint swelling and pain and also limits the joint damage (6).

1.1.2 Criteria for RA

The 1987 American college of Rheumatology (ACR) classification criteria for RA are: morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, positive serum rheumatoid factor(RF), and radiographic changes. Definite RA is diagnosed if more than 4 of these 7 criteria are fulfilled (7). In 2010 ACR/EULAR criteria was framed to diagnose RA in early stages of the disease, the new criteria aims to prevent the structural damage by initiating therapy in earlier stage (8). The new criteria focus on the joint involvement (number and sites involved in the joint), serologic abnormality (RF and ACPA), acute-phase reactants and symptom duration (8). Presence of the autoantibodies against the citrullinated antigen (ACPAs) and the autoantibodies against the Fc part of self-IgGs known as rheumatoid factor (RF) can be used to sub-divide RA patients. Based on the autoantibody positivity the patients can be classified as seropositive and seronegative RA, which differ from one another in terms of their respective disease courses (9).

1.1.3 History of serological markers in the clinical diagnosis of RA

RF was initially identified as an antibody against serum gamma-globulin in 1940 (10). Later, it was found that these antibodies are detectable in other infectious and autoimmune diseases (11). In 1948 RF was described in patients with RA (11) and from 1987, it has been included in the clinical diagnosis of RA (12).

In the early 1960, it was found that the antibodies present in the RA-sera reacted specifically to keratohyalin granules and later this property was employed in the serological test popularly known as anti-perinuclear-factor (APF) test. (13) After several years of research the antigen was found to be modified filaggrin present in the fully differentiated epithelial cells where the aminoacid arginine was replaced by citrulline (14). These findings were later confirmed by the high specificity binding of the RA-associated autoantibodies to the citrullinated filaggrin or linear citrullintaed peptides but the sensitivity was low (15). Later, citrullinated peptides were changed to cyclic citrullinated peptides (CCP), resulting in an improved sensitivity of 68% and specificity of 98% (16). It was followed by the development of CCP2 test and is being used as standard ELISA in the clinics for ACPA detection (17).

1.2 Etiopathogenesis of the longitudinal development of seropositive RA

Current understanding of the seropositive RA pathogenesis, suggests that the development of RA-associated autoantibodies in individuals with specific genetic background leads first to unspecific symptoms (such as pain and fatigue) that later culminate in chronic joint inflammation, in some but not all of these individuals. This section discusses the current state of the art regarding the longitudinal development of seropositive RA (5) which is schematically represented in figure 1.

1.2.1 Genes

Genetical background can be an important risk factor for the development of RA and it can also influence disease severity. Epidemiological studies has shown an association between human leukocyte antigen (HLA)-DRB1 alleles and RA (18, 19). Shared epitope (SE) consists of five aminoacids from the position 70-74 in DRß chain is encoded by the HLA-DRB1 gene and involved in the antigen presentation by T cells (19). SE is associated with the increased diseases severity and erosions (19, 20). Furthermore, the presence of SE is linked to the predisposition towards ACPA positive RA (21) and this contribution is only linked to the ACPA development but not to the diseases progression (22). Studies on the non-MHC associated genes have also been reported to associate with RA. Risk alleles are Protein

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tyrosine phosphatase non-receptor 22 (PTPN22) (23) is associated in both seropositive and seronegative RA patients (24), whereas Cytotoxic T-lymphocyte protein 4 (CTLA-4) (25) and peptidyl-arginine deiminase type 4 (PADI4) (26) are associated with only seropositive RA patients.

1.2.2 Environmental factors

RA is characterized by complex gene–environment interactions (27). Long-term smoking, particularly in individuals carrying SE in the MHC molecules, is strongly associated with the risk of developing seropositive RA (28) (29). Other factors like silica and textile dust exposure are also associated with the ACPA-positive RA development (30, 31).

In a case control study involving subjects exposed to silica and rock drilling have 1.5fold and 2.5fold increased risk of developing ACPA positive RA (31). Exposure to silica with current smoking additively increased the risk of the developing RA (31, 32).In a Malaysian-based case control study, textile dust exposed females have an increased risk of developing ACPA positive RA by an odds ratio(OR) of 2.8 (33). OR was even higher in those individuals that have both the shared epitope and textile dust exposure (33). A U.S and Swedish cohort study based on the exposure to air pollutants such as particulate matter (PM_{10} and $PM_{2.5}$) and gaseous pollutants (SO_2 and NO_2) showed no association to the risk of developing RA (34). On the contrary, a study on exposure to the traffic pollution suggests that the woman living as close 50m to the major roads have increased risk of developing RA compared to those living farther (35).These findings have led to the hypothesis that mucosal surface in the lungs might be involved in triggering RA development.

1.2.3 Environmental challenges lead to mucosal immune activation

Environmental challenges (such as smoking) directly affect the mucosal surfaces of the body. These mucosal surfaces are trying to maintain an equilibrium between external challenges (either negative of positive) and the body homeostasis, through innate and adaptive immune mechanisms (36). A recent study in the ACPA positive patients with early untreated RA demonstrated presence of lung abnormalities on high resolution computed tomography (HRCT) (37). This was associated with signs of immune activation, increased expression of citrullinated proteins and ACPA enrichment (38). ACPAs are also enriched in the sputum of the individuals at-risk of developing RA and correlate with other inflammatory parameters detected in the sputum (39, 40). These observations give support for an initial immune attack

leading to tolerance breaching and immune activation at extra-articular sites, particularly at mucosal sites.

1.2.4 Systemic autoimmunity prior to disease onset

Following their appearance at mucosal sites, ACPAs can be present in the circulation several years, together with RF antibodies, before the onset of systemic inflammation (41). The presence of these autoantibodies is associated with an increased risk of developing RA, and the constantly evolving ACPA fine specificities lead to epitope spreading and a broad range of recognized targets by the onset of RA (41, 42). As discussed later in the thesis, increase in ACPA production and diversification of the recognized targets typically accompanies the initiation of RA suggesting a potential role for ACPAs in the progression towards the disease although the cause and consequence relationship between the increase of ACPA responses and systemic inflammation is yet to be fully understood (43, 44). Moreover, ACPAs against different target molecules can increase or remain at low level during RA onset suggesting that certain ACPA specificities might be more important for disease progression than others (45). In addition, different ACPA isotypes can be present in the circulation with IgG ACPAs being the most predictive for disease onset and the number of detectable ACPA isotypes (IgG1, IgG2, IgG3, IgG4, IgA or IgM) by the initiation of RA has been shown to correlate with radiographic damage in the course of the disease (40, 46). Recent analysis of a potential hierarchy within ACPA responses against exogenous or endogenous antigens being more relevant for progression towards RA has not detected any such difference (47).

It is important to note that although the presence of ACPAs alone is not sufficient for RA development, together with genetic risk factors (such as HLA SE), environmental risk factors (such as smoking) and the presence of joint pain, ACPA positivity indicates a high chance for eventual progression towards RA.

1.2.5 Chronic joint inflammation

Yet unknown mechanisms are responsible for the transition between systemic autoimmunity and joint inflammation and these mechanisms still remain to be identified. Once joint inflammations can be detected in form of swollen and painful joints in a seropositive individual, a diagnosis of RA can be made in a large majority of the cases. This phase is characterized by development of chronic synovitis with increased infiltration of mononuclear cells and lymphocytes is reported into the synovial tissue and synovial fluid (SF) of the RA patients (5) as well as the activation and expansion of macrophages (MΦ) and fibroblast-like

cells that compose the synovial membrane (48). The activated stroma and the infiltrating immune cells release an array of inflammatory mediators that increase immune activation, chemotaxis, osteoclast (OC) development and angiogenesis in the newly expanded synovial tissue. ACPAs and RF antibodies can further accelerate immune activation through triggering cytokine production in MΦ and dendritic cells (DCs) through immune complex-mediated triggering of activating FcRs on these cells (49). The chronic inflammation fuels cartilage and bone destruction due to the increase of OC differentiation and through the invasion of cartilage by erosive synovial fibroblasts (50, 51) (52)
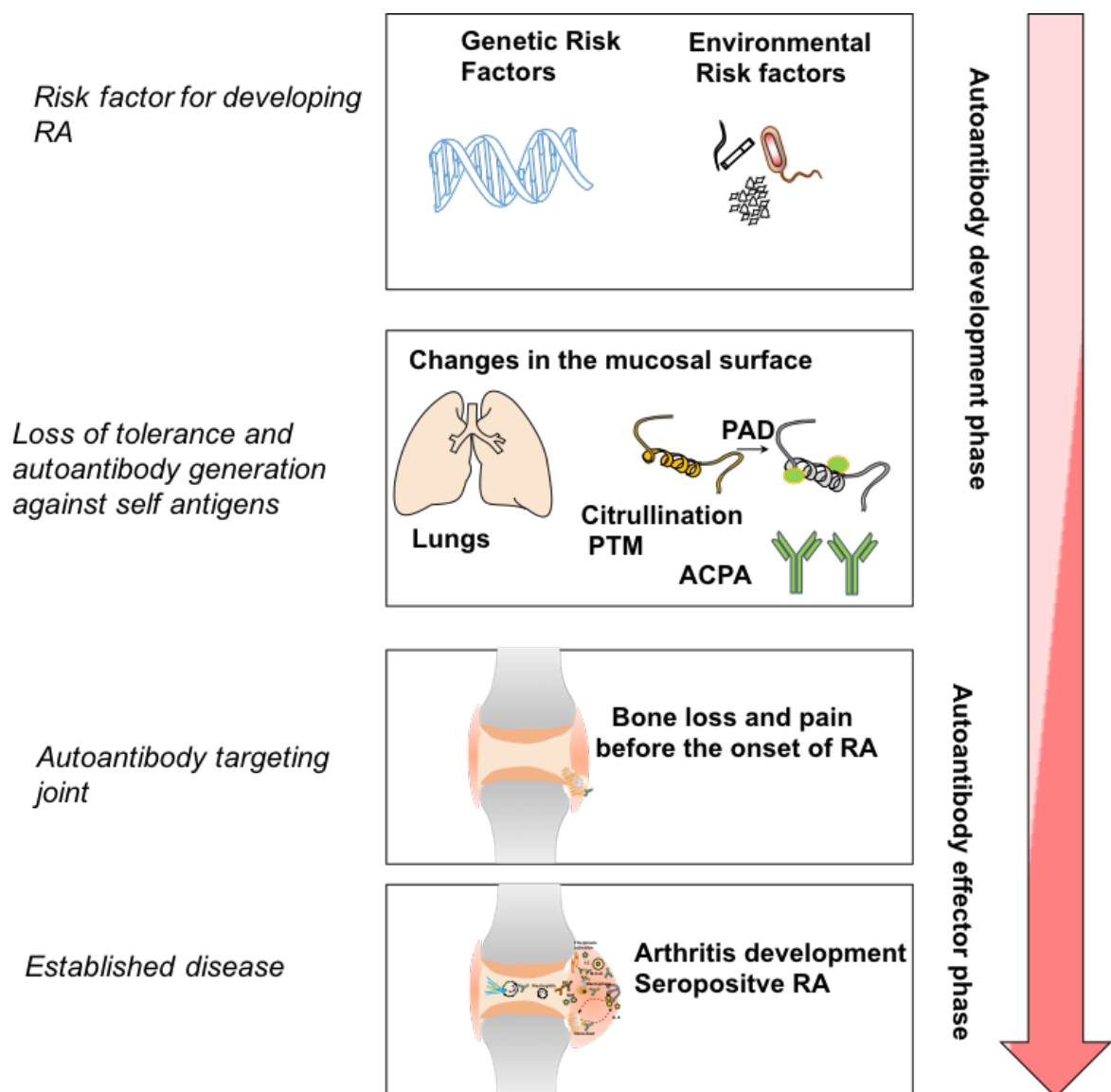
Figure 1: Longitudinal view of the seropositive rheumatoid arthritis

Genetic and environmental factors have vital role in the development of RA. Genetically susceptible individuals challenged with environmental factors as smoking, textile dust and microorganisms could trigger the local inflammation and immune activation that results in the loss of tolerance against the self-proteins and leads to the autoantibody generation by the

adaptive immunity. Autoantibodies increase in the titre and undergo epitope spreading. Over time, these autoantibodies gradually increase in titre and epitope spreading happens which occurs before the onset of the disease. Studies have linked the autoantibody positivity to bone loss and pain. During this period, a minor challenge or infection could lead to chronic inflammation and arthritis development.

1.3 Tolerance breaking and immune activation in RA

As mentioned above tolerance braking in RA could be initiated at mucosal sites and posttranslational modifications induced by exposure to air pollutants such as smoking are believed to play an essential role.

1.3.1 Role of post-translational modifications

Post-translational modifications (PTMs) lead to covalently modified proteins often with altered biochemical properties that can affect protein-protein interactions or enzymatic reactions (53). There are around 200 different types of PTMs identified (54), out of these citrullination, carbymylation, acetylation and malondihyde modification have been studied in RA (55) due to detectable autoantibody responses against the modified proteins in RA (55). As autoimmunity against citrullinated modified proteins is one of the most frequent detected and specific autoimmunity in patients with RA, we have in the current thesis focused on investigation of citrullination as a prototype PTM.

1.3.1.1 Citrullination

Citrullination is the conversion of peptidyl arginine to peptidyl citrulline and was first defined by Rogers and Simmonds in 1958 (56). This modificaiton renders the strong positive charge in arginine into neutral citrulline (57). The change in the charge of the proteins leads to decrease in the mass of the citrullinated protein by 1Da (57). Citrullinated proteins are present in the cytoplasm, nucleus, plasma membrane and mitochondria of a cells (58). Citrullination can occur in various tissues under both physiological and pathological circumstances (59).
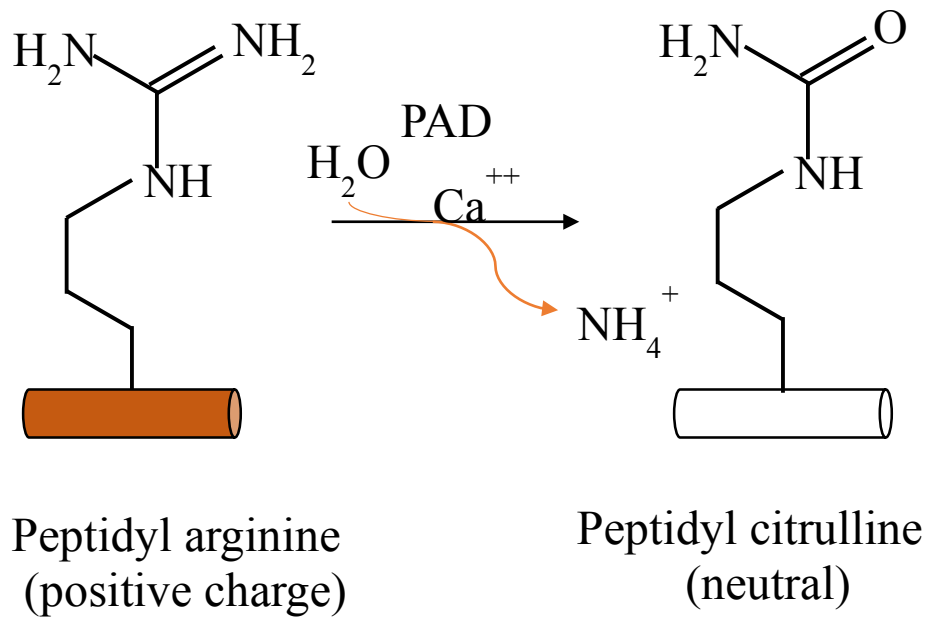
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Figure 2: Process involved in Citrullnation

This figure shows the hydrolytic reaction by PAD enzymes that converts the peptidyl arginine into peptidyl citrulline in presence calcium. The conversion results in the change in the charge from positive to neutral.

During late differentiation phases of keratinocytes, citrullination of their main intermediate filament protein, the cytokeratin, enables structural changes that alters the spectrum of cytoskeleton-linked membrane proteins (57, 60). During the development of the brain, in the first two years of life an elevated citrulination of myelin basic protein allows increased neuronal plasticity, which is followed later by drastically reduced citrullination of the same protein (60). Histone citrullination regulates the chromatin structure, and it is required for the pluripotency of stem cells (61). Vimentin is another important intermediate filament protein that is present in various cell types and plays important roles in adhesion and cell migration (62). During apoptosis, citrullination of vimentin reduces the isoelectric point of the head domain, decreasing the capacity of the protein to polymerise, which potentially contributes to the morphological changes occurring in apoptotic cells (57). Citrullination alters the immunological functions of the chemokine IL-8(CXCL8), through interfering with the maturation of this chemokine by thrombin-mediated proteolysis and thereby decreasing the neutrophil recruiting capacity of the molecule (59).

Increased protein citrullination has been associated with autoimmune diseases, inflammation or trauma. In multiple sclerosis patients increased citrullination of histones is reported in the

normal white matter and in animal models demyelination. In the same report TNF was identified as the mediator that induced citrullination by inducing PAD4 (63). In RA, citrullinated protein have been identified in the joints, including several known target for ACPAs (e.g. vimentin, enolase, fibrinogen). Smoking has been associated with increased citrullination, which is likely to contribute to an increased risk for developing ACPA-positive RA. Traumatic brain injury leads to increased citrullination in certain regions in the brain, occurring mostly in astrocytes (64).

1.3.1.1.1 <u>PAD enzymes</u>

Peptidyl arginine deiminases (PAD) are enzymes responsible for citrullination. PAD enzymes were identified in 1977, they can citrullinate arginine in the proteins but cannot citrullinate methylated arginine in the context of the protein (65). In humans there are five PAD isozymes, they are PAD1, PAD2, PAD3, PAD4 and PAD6, all the PAD isozymes except PAD6 has *in-vitro* activity. PAD2 and PAD4 are distributed in wide range of tissues and cells has been implicated in several diseases like RA, cancer, ulcerative colitis, Alzheimers diseases and multiple sclerosis (58).

Early studies on the catalytic activity of PADs has demonstrated that high amount of calcium (5-10mM) is required for the PAD enzymes to get activated in vitro *(66, 67).* This extremely high concentration is difficult to achieve in the normal physiology. Recently, anti-PAD4 (identified by cross-reactivity with PAD3) autoantibodies has been described, these antibodies can increase the catalytic efficiency of PAD4 decreasing the requirement of the calcium to the physiologic range. PAD activity and expression has been detected in the SF and peripheral blood monocytes and macrophages (68) of RA patients (69). In relation to the PAD activity SF calcium levels were reported to be sufficient to citrullination of the proteins (69). Interestingly, monocytes can upregulate PAD2 and PAD4 expression when they interact with the activated T-cells, providing a source for increasing levels of PAD enzymes (70). It has been recently shown that the TNF can interact with PAD4 to increase the citrullination thereby increase the autoantibody production against native and citrullinated targets. Moreover, TNF transgenic mice lacking PAD4 showed decreased autoantibody production and inflammatory arthritis (71). Expression of the PAD enzymes and citrullinated proteins are increased in the inflamed tissue sites in the patients with autoimmune diseases and colitis (72). Citrullinated vimentin stimulated the proliferation of the fibroblasts from RA patients, in addition stimulating proinflammatory cytokine secretion (73). The relation between PAD overexpression and inflammation has been implicated but the mechanism behind the increased inflammation with increase in PAD has not been described until recently. A report

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on PAD4 in neutrophils suggests that PAD4 can citrullinate NF-kB p65 thereby enhancing their nuclear localization and inducing the inflammatory cytokines (74). These findings suggests that PAD mediates inflammation through their capacity to regulate transcription factors and through other unknown mechanisms .

As abnormal PAD activity has been implicated in several diseases state, PAD blocking agents have been suggested as potential therapeutic agents in several animal models including RA (75-78). Recently, it has been shown that targeting PAD enzymes increased apoptosis of the inflammatory cells in the colitis mice model, suppressing the colitis (75). In malignant tumours, PAD enzymes are overexpressed downregulating the tumour suppressor p53 expression, inhibiting PAD enzymes in xenograft tumour model proved to be effective in suppressing the tumour growth by activating the tumour suppressor gene expression (79). In experimental autoimmune encephalomyelitis (EAE), an animal model for CNS demyelinating disease, hypercitrullination by PADs correlates with the diseases activity, blocking the PAD enzymes before the initiation of the autoimmunity prevented the disease by suppressing the autoreactive T cells (80). Interestingly, Cl-amidine a PAD inhibitor treatment of murine collagen induced arthritis (CIA) model lead to decrease in synovial and serum citrullination, followed by decrease in disease activity by 50 % and reduced IgG2a anti-mouse collagen antibodies (81).

1.3.2 Role of Innate immunity

The innate immune system is composed of several soluble and cellular components, which are rapidly activated in response to danger signals that indicate tissue damage or the presence of invading pathogens (82). Activation of the innate mechanisms provides a first line defence for the host organism and it also sets the stage for the adaptive arm of the immune system through and array of inflammatory/co-stimulatory mediators that facilitate T cell and B cell responses (36). Innate immune mechanisms are crucial for the initiation of autoimmune responses as well, with triggering mechanisms potentially including endogenous compounds, environmental stimuli or microbial factors (83, 84). Smoking and other environmental insults of the airway mucosa are well-known risk factors for seropositive RA and these signals might broadly influence the airway epithelial and antigen presenting cells by causing oxidative stress or through compounds that directly regulate these cells (83, 85). Interestingly, cigarette smoke induces approximately 100X increase in airway endotoxin exposure as compared to smoke-free environment (86, 87). The activation of pattern-recognizing receptors, such as Toll-like receptors or inflammasome components, in response to cigarette smoke can result in

the production of inflammatory cytokines and chemoattractants by cells in the airways (36). Moreover, MΦs and DCs have been shown to respond with altered phenotype and functionality to cigarette smoke, with both inhibitory and stimulatory effects described (88, 89)(78). These cells might play a particularly important role in the initiation of the anti-citrullinated protein responses due to persistent steady state citrullination occurring in their autophagic compartments and a subsequent MHC-II dependent presentation of citrullinated peptides on the cell surface (90). In addition to a potential activation of antigen presenting cells in the airways, cigarette smoke could further contribute to the initiation of citrullinated protein-specific immune responses through increasing PAD enzyme expression and the amount of citrullinated proteins in the lungs (89).

Neutrophil granulocytes constitute a crucial first-line defence against extracellular pathogens due to their phagocytic and intracellular killing activities and release of neutrophil extracellular traps (NETs), a network of externalised chromatin, which can immobilise and kill several pathogens (91). NET production requires PAD4 activity and histone hypercitrullination, suggesting that NETosis might play an important role in providing citrullinated proteins to the adaptive immune system (92). In addition, the periodontal pathogen Aggregatibacter actinomycetemcomitans (Aa) has been shown to induce hypercitrullination in neutrophils, likely through the pore-forming toxin leukotoxin A, which activates PAD enzymes by inducing Ca2+ influx in the cells and also facilitates a subsequent netosis (93). Interestingly, the association of HLA SE expression and RA-associated autoantibodies was restricted to Aa-positive patients in this study, suggesting the importance of this neutrophil-bacterium interaction in the initiation of seropositive RA.

Innate immune cells play important roles in established RA as well, through contributing to the production of inflammatory mediators and the presentation of autoantigens to the adaptive immune system. Monocytes, MΦs and DCs are recruited to the inflamed joints and can differentiate into OCs contributing to bone damage . Immune mediators turn fibroblasts of the synovial membrane into an expanding inflammatory stroma that erode cartilage, present antigens to the infiltrating T lymphocytes and release an array of cytokines, matrix degrading enzymes, chemoattractant, angiogenic or osteoclastogenic compounds (94). Therapies targeting key inflammatory cytokines are among the most efficient approaches tested so far to ameliorate RA, indicating the profound importance of these pathways in disease pathogenesis (95) .

1.3.3 Role of adaptive immunity

Genes encoding HLA-SE or PTPN22, a phosphatase that regulates T and B cell receptor signaling, are the most prominent susceptibility loci that have been associated with ACPA-positive RA indicating a prominent role for adaptive immunity in RA (9). Protein citrullination, due to converting a positively charged arginine to a neutral citrulline, has been shown to increase binding affinity of several antigenic peptides to the positively charged P4 pocket in HLA-DRB1 molecules, proposing a model where a preferential presentation of citrullinated autoantigens could trigger autoreactive T cells responses in SE-positive individuals (9). Additionally, citrullination affects proteolysis and consequently alters the repertoire of the presented peptides (96). Moreover, in some cases, citrullination of the enolase protein was likely to affect interaction with the TCR of autoreactive T cells instead of MHC binding (97). T cell reactivity to citrullinated autoantigens facilitate B cell responses and the subsequent ACPA production. ACPAs have been detected in the airways as well as in the serum of individuals at risk for developing RA and the work presented in this thesis focused largely on the role of these antibodies in the initiation of RA due to mechanisms contributing to bone erosion and pain. A rapid increase in epitope spreading and intensity of ACPA responses typically marks and then follows the onset of RA suggesting an active contribution of these antibodies in disease development. Interestingly, ACPAs targeting certain citrullinated targets expand more robustly, whereas others remain permanently at low level suggesting a differential contribution of certain ACPA specificities to RA progression (45). Indeed, association with genetic risk factors or smoking was more a characteristic for patients with specific patterns of ACPA reactivities, instead of anti-citrulline responses in general (98, 99). Several monoclonal ACPAs, representing single autoreactive B cells or plasma cells obtained from RA patients, have been recently isolated and characterised in detail (100, 101).These studies have also suggested that in spite of their prominent cross-reactivity, individual ACPA clones can be associated with unique cellular targets and pathological mechanisms. In addition to directly triggering cell surface receptors, ACPAs can contribute to inflammation and bone erosion by forming immune complexes and activating FcγR molecules on MΦs, Dcs or developing OCs (102-104). ACPA immune complexes have been shown to trigger cytokine production in MΦ, which effect was further stimulated by the presence of RF antibodies (49, 105, 106). Importantly, in addition to the constant evolution of ACPA fine specificities, glycosylation of the antibodies has also been shown to alter during disease progression, influencing binding capacity to Fc and putative lectin-type receptors and, consequently, the cellular effects triggered by ACPA-containing immune complexes (103) .

1.4   Disease propagation: pain, joint inflammation and bone destruction

Following tolerance breaking and immune activation antibodies are present in the peripheral blood for several years before development of pain, joint inflammation and bone destruction.

1.4.1   Pain

Pain, an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage and it represents one of the main reasons why patients seek medical attention (107). Pain can be classified as acute and chronic pain and based on the underlying pathophysiology it can be divided to nociceptive and neuropathic pain. Pain without identifiable physical or physiological source can be classified as idiopathic pain. Peripheral sensitization due to inflammation or injury can increase pain sensitivity for a long period of time (108). This can be due to the hyperalgesia, a hallmark of inflammation, which is increased pain sensitivity to normal pain stimuli and allodynia, which is pain in response to otherwise non-painful stimuli (109). Peripheral sensitization is mediated by cytokines and other inflammatory mediators typically released at sites of injury. Cytokines can also be released by astrocytes and glial cells leading to central sensitization. Acute pain involves the nociceptors in a localized tissue (110) whereas chronic pain involves both the nociceptive stimuli and the central nervous system sensitization, causing a reduction in the general pain threshold that leads to appearance of the hypergelsia and allodynia (111).

Pain is a major complaint of RA patients that often develops already before disease onset (112). Presence of pain and specifically a certain type of pain (often describe as clinically suspect arthralgia) might help the rheumatologists in identifying individuals having a high risk for developing RA (113). A longitudinal study has indicated that ACPA-positive individuals having arthralgia progress more rapidly towards RA than ACPA-negative individuals (114). The presence of pain already before the onset of joint inflammation suggests that the mechanisms leading to pain might, at least partially, be dissociated from inflammation (115). Once the disease is diagnosed, pain is carefully followed up and treatment of pain is a major goal for therapy in RA. A majority of the patients will experience pain improvement as the disease is controlled and they enter clinical remission but a not negligible amount of pain remains reported, known as remaining pain (115).

Pain after RA onset can be related to the inflammation in the synovium (116). Sensory neurons innervate the knee joints and send signals to the central nervous system (109). Inflammatory mediators released in the joint can directly act on the peripheral sensory neurons and induce pain (109). In experimental arthritis, pro-inflammatory cytokines,

including TNF, IL-1ß, IL-6 and IL-17, have been shown to sensitize the peripheral neurons (117). ACPA immune complexes might contribute to arthralgia via FcγRs present on primary sensory neurons (118). Interestingly, TNF blocking therapy has shown to produce analgesic effect before reducing the local inflammation, suggesting the TNF inhibition affects the central nervous system (119). Accumulating evidence suggested that the pain in RA is not only dependent upon peripheral sensitization but it can also lead to altered central pain processing (120).

1.4.2 Joint Inflammation

Local accumulation of immune cells and cytokines leads to a synovitis. Normal synovial membrane is thin and consists only few cellular layers specialized columnar FLSs with few scattered MΦ or lymphocytes (121, 122). In the rheumatoid synovitis the lining layer becomes hyperplastic and might protrude into adjacent bone resulting in pannus formation while the sublining layer is characterized by massive cell infiltration and new blood vessel formation (123, 124). Pannus is a destructive tissue that is present in the interface between the synovium, cartilage and bone is clearly a feature of the erosive disease (125). Synovial needle arthroscopy is a technique used to obtain the synovial biopsy of the rheumatic patients (126). The synovial biopsy gives information on both the macropscopic and microscopic changes in the tissue (121). The macroscopic changes demonstrate the inflammation and hyperplastic synovial villi and the microscopic changes in the synovium reveals the accumulation of the cells with neo-vascularisation (121).

The inflamed synovial tissue in RA contains heavy infiltration of the cells such as monocytes, fibroblasts, MΦ, T and B lymphocytes, plasma cells, neutrophils, mast cells, DCs and natural killer cells enter through the increased number of highly activated post-capillary venules from the circulating blood (121, 127, 128).The MΦ/monocytes are abundant and express CD14, CD68 and CD163 as their phenotypic markers. The synovial MΦ are the major source of cytokines causing local inflammation and joint destruction (129). T cells express CD3 surface markers and are mainly CD4 positive cells found in RA synovium either as aggregates or infiltrates in the sublining layer (130). Fibroblasts are resident cells able to secrete cytokines, enzymes and acids that contribute to the invasive nature of the rheumatoid synovium. T and B cells, plasma cells organize to form aggregates produce autoantibodies associated with RA thus involving in the disease pathogenesis (131). The infiltration of the immune cells leads to increased demand of oxygen resulting in hypoxia, which directly relates to macroscopic and microscopic inflammation, cell migration and cytokine production (132). Suggesting that the

hypoxia has a role in initiating the synovial inflammation (132). Changes in the synovial tissue has been used to predict the treatment response to the conventional and biologic DMARDS and to identify the drug induced changes in the synovial architecture (133, 134). SF reflects the pathological changes in the synovial tissue which can be seen as increase in the protein content in the inflamed state in comparison to the steady state (135). The changes in the SF is due to the increase in the cellular infiltration, synovial hyperplasia and increased vessels permeability (121). These changes inturn increase the levels of pro-inflammatory cytokines in the SF and increased local production of the ACPAs which are capable of perpetuating the joint inflammation.

Cytokines are non-structural protein or glycoproteins secreted by the immune cells involved in cell signaling in an autocrine, paracrine or endocrine fashion. They mediate inflammation, tissue repair, cell growth, fibrosis, angiogenesis and immune response (136). MΦ and fibroblasts are major source for cytokines production in the rheumatoid synovium (136). Previous studies on RA synovial cultures showed increased production of IL-1, when compared to synovium obtained from osteoarthritis pateints (137). CD4+ helper T-lymphocytes produce two different cytokine profiles namely Th1 (cell mediated response) and Th2 (Humoral mediated response) (138). RA synovium has mainly a Th1 cytokine profile. However, some studies provide evidence to Th2 (IL-6 and IL-10) mediated inflammatory response (139) (140). Recently a new type of T cells called Th17 (IL-17) have been identified and are currently considered as important players in propagation of local inflammation and bone destruction (141). IL-1 and TNF-α are the major proinflammatory cytokines involved in the pathogenesis of the RA and act by inducing local inflammation, increasing inflammatory cell adhesiveness, promoting angiogenesis and bone resorption (140) (137). In RA, cytokine released in the inflamed tissue induces synovial fibroblasts to produce M-CSF in the joints (142). Increased infiltration of the MΦ is synovia of the active RA patients (143)and treatment with the biologic drugs like anti-TNF drugs decreases influx of the cells from the synovia (144). Drugs targeting pro inflammatory cytokines are effective in patients with RA (145) (146).

1.4.3 Bone loss

Bone loss is a central feature of RA resulting from an imbalanced bone formation and bone erosion. This section focuses on the bone loss in RA, role of osteoclast in bone loss and the link between the immune system and bone.

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1.4.3.1 *Types of bone loss in RA*

Periarticular bone loss is characterized by a reduction of the trabecular size and number in the region close to the joint (147). Periarticular osteopenia detected on X rays has long been a well-recognised feature of established RA and part of the old classification criteria for RA. A large array of pro-inflammatory cells and mediators present in the inflamed synovium contribute to periarticular bone loss by both activating bone loss and inhibiting the new bone formation (148) (149) (150) (151). More recently, periarticular bone loss has been however reported already at disease onset (152) and in seropositive individuals at risk for developing but not yet having disease (152), suggesting that bone loss might be at least partially uncouple from inflammation.

Systemic bone loss, osteoporosis, with an increased fracture risk is common in patients with longstanding RA (153) (154). The factors that likely contribute to the systemic bone loss include reduced physical activity, systemic effects of the inflammation and the intake of glucocorticoids(147) (155). It has been reported that as many as 25% of the RA patients show a reduced bone mineral density of the spine or hip already before treatment and that around 10 % can be diagnosed with osteoporosis already in these early stages (156). Recent findings on the ACPA positive individuals at risk of developing RA, have demonstrated altered bone mineral density (BMD), decrease in the bone volume and cortical bone thickness, in comparison to the ACPA negative individuals (152). Another study on the longitudinal follow-up of ACPA positive individuals confirm the findings in the pre-clinical phase of the disease (157).

1.4.3.2 *Osteoclasts the bone destroying cells*

Two types of cells are essential for bone biology: osteoblasts - the bone forming cells and osteoclasts (OC)- the bone resorbing cells. The osteoblasts are derived from the mesenchymal/stromal cells and function as bone lining cells. These cells are essential for secretion and mineralization of the bone matrix (158). Osteoblasts continuously replace the new matrix and mineralize the bones that are resorbed by the OCs. Maintaining the joint integrity needs balance between anabolic and catabolic processes affecting the bone. Normally the human skeleton is remodelled constantly, with around 10% of the total bone content replaced every year in adult vertebrates (159). It is estimated approximately there are around 2 million microscopic sites, where the bone remodelling happens to maintain the skeletal integrity (159). OCs are the only cells capable of resorbing of bone. They are present

in the resorption lacunae and interface between bone and synovia(160). Studies on OC activity have suggested the impaired OC activity can lead to the reduced regeneration of the bone with age (159).

OCs attach to bone using αVß3 integrin and attachment results in sealing zone formation which separates the resorptive pit from the extracellular surface. Next, is the ruffled border formation representing the active state of the OCs. OCs utilize the proton pumps to secrete the hydrochloric acid which acidifies the resorptive environment and activates the proteolytic enzymes like cathepsin K dissolving the bone. The ruffled borders are also used by OCs to remove the degraded products by endocytosis through vesicular formation and then exocytosed into the extracellular surface by the atypical membrane surface (159).
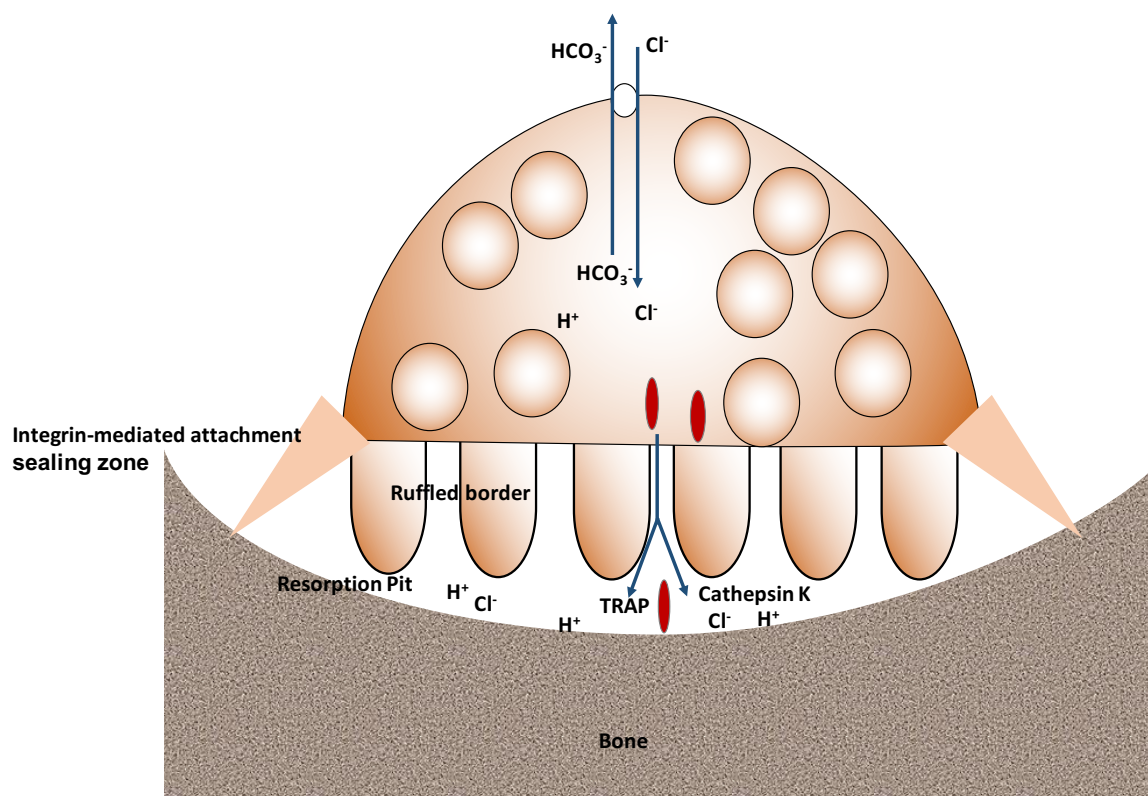

Figure 3: Process of bone resorption

Bone resorption by mature OCs is dependent on the attachment of the OC along with the sealing zone formation on the surface of bone, followed by the release of the acids and proteolytic enzymes into the lacunae lysing the bone.

OCs can be identified by their distinguishable multinucleated morphology and they are known to express the tartrate resistance acid phosphatase (TRAP) in the cytoplasm, an iron containing enzyme common to bone and the immune system. The presence of TRAP has also been reported in monocytes, MФ and DC but in serum TRAP exist in two isoforms, 5a and b

formed by the posttranslational modification of a single gene product (161). MΦ and DC releases TRAP in the isoform 5a whereas 5b is predominantly expressed by the OCs (161). TRAP enzyme activity can be detected in the supernatant of active OC cultures and it co-relates with the activity and resorption area (162).Investigation on the OCs led to the identification of proteolytic enzyme cathepsin K(163) and calitonin R(160) considered as more OC specific markers.

Reports suggest that TRAP is transported in the vesicles along with the matrix degradation products to the basolateral surface from which where they are released (164). The resorbing activity carried out by the OCs is due to the reactive oxygen species produced by TRAP released from the ruffled borders of the OC (162, 165). The oxygen radicals produced by TRAP in MΦ help in antigen processing before presentation (165). Studies on the normal bone homeostasis and calcium levels in the body have identified Vitamin D and hormones as major contributors for the bone remodeling.

1.4.3.1 Role of Vitamin D and hormones in osteoclastogenesis

Initial studies, on the Vitamin D demonstrates its capacity to mineralize bone, later it was found that the active form of the vitamin $D_3(1\alpha,25(OH)2D3)$ stimulates bone resorption (166). Mice bone marrow mononuclear cells cultured in the presence of $1\alpha,25(OH)2D3$ differentiated into functionally active TRAP positive OCs (167). It was also shown using an organ culture system with rat bone increased release of prelabelled ^{45}Ca (calcium) can be induced by $1\alpha,25(OH)2D3$ (168).

Parathyroid hormone (PTH) can activate OCs indirectly by triggering their receptors expressed in osteoblasts, in turn leading to the increased expression of pro-osteoclastogenic factors (169). Continuous exposure to the PTH leads to increased bone loss whereas intermittent treatment with PTH increased BMD (169). The anabolic effects of intermittent PTH can be related to the increase in the insulin-like growth factor (IGF) that augments the proliferation of the osteoblasts (170). Both PTH and Vitamin D work together to mobilize the Ca from the bone and conserve the Ca loss from the body(166). Human body is dependent on the Ca release for the function of the cells and is regulated by the bone. Foetal growth and lactation in females is also dependent on the mobilization of the Ca (159).

Calcitonin, a hormone released by the thyroid gland can regulate osteoclastogenesis directly by binding to its receptors expressed on the OCs. It can arrest bone resorption by targeting ruffled borders followed by cell retraction (171). These findings suggest that hormones and vitamin D has a major role in bone remodeling and calcium homeostasis.

1.4.3.2 The link between OC and inflammation

In a normal individual, a fine balance exists between bone forming cells of mesenchymal origin (osteoblasts) and bone resorbing cells developed from hematopoietic precursors (osteoclasts) (172). In RA, the balance between bone formation and bone resorption is lost leading to bone destruction. This imbalance is partially mediated by pro-inflammatory cytokines. Chronic inflammation in RA contributes to the priming of the hematopoietic precursor cells that become more susceptible to OC formation (173). Formation of OC requires either differentiation of monocytes or transdifferentiation of immature DCs in the presence of essential factors such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) (174)'(175). This process is further sustained by pro inflammatory cytokines such as tumor necrosis factor alpha (TNF), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (176) abundantly produced by local accumulation of immune cells in the inflamed synovia.

M-CSF is a glycoprotein that binds to the Colony stimulating factor 1 receptor (CSF-1R) encoded by c-fms proto-oncogene (177). M-CSF supports the OC differentiation by up regulating RANK expression (178), inducing OC precursors proliferation and survival (179) (160, 177). M-CSF induced activation of the OCs is dependent on the extracellular signal-regulated kinases (ERK) and Phosphatidylionositol 3-Kinase (PI3K) pathway (180). The importance of the M-CSF has been demonstrated by using the mice lacking the CSF-1R which exhibits osteopetrotic phenotype (180). RANKL is a TNF super family member that promotes OCs differentiation, expressed by the immune cells (T and B cells) (181), osteoblasts (182), hypertrophic chondrocytes(183), osteocytes(184) and fibroblasts like synoviocytes (RA-FLS) (185). RANKL binds to its receptor RANK expressed on the surface of OC precursors and promotes the maturation and activation of OCs (186). The binding of RANK to RANKL initiates the intracellular signalling through TRAF6 (TNF receptor associated adaptor factor 6), activation of the nuclear factor kappa B (NF-κB) leads to induction of the nuclear factor of the activated T cells c1(NFATc1) which is the master regulator of the terminal OC differentiation (180). A natural decoy receptor called osteoprotegrin (OPG), able to bind RANKL, counters its effect (187). OPG binding to RANKL down regulates RANKL signaling. OC differentiation is regulated by the balance between the OPG and RANKL (188).

Apart from the essential cytokines, osteoclastogenesis is influenced by the several other cytokines that can either promote or inhibit osteoclastogenesis through their direct and indirect effects (189). Cytokines like TNF, IL-1, IL-6, IL-8, IL-15, 1L-17, IL-23 can increase

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the osteoclastogenesis by mostly inducing RANKL production on other cells in the synovium, except TNF, IL-6 and IL-8 which can directly influence OC formation (189-191). On the contrary, IL-4, IL-10, , IL-12, IL-18, IL-27, IL-33 and GM-CSF can potently inhibit osteoclastogenesis (189, 190).
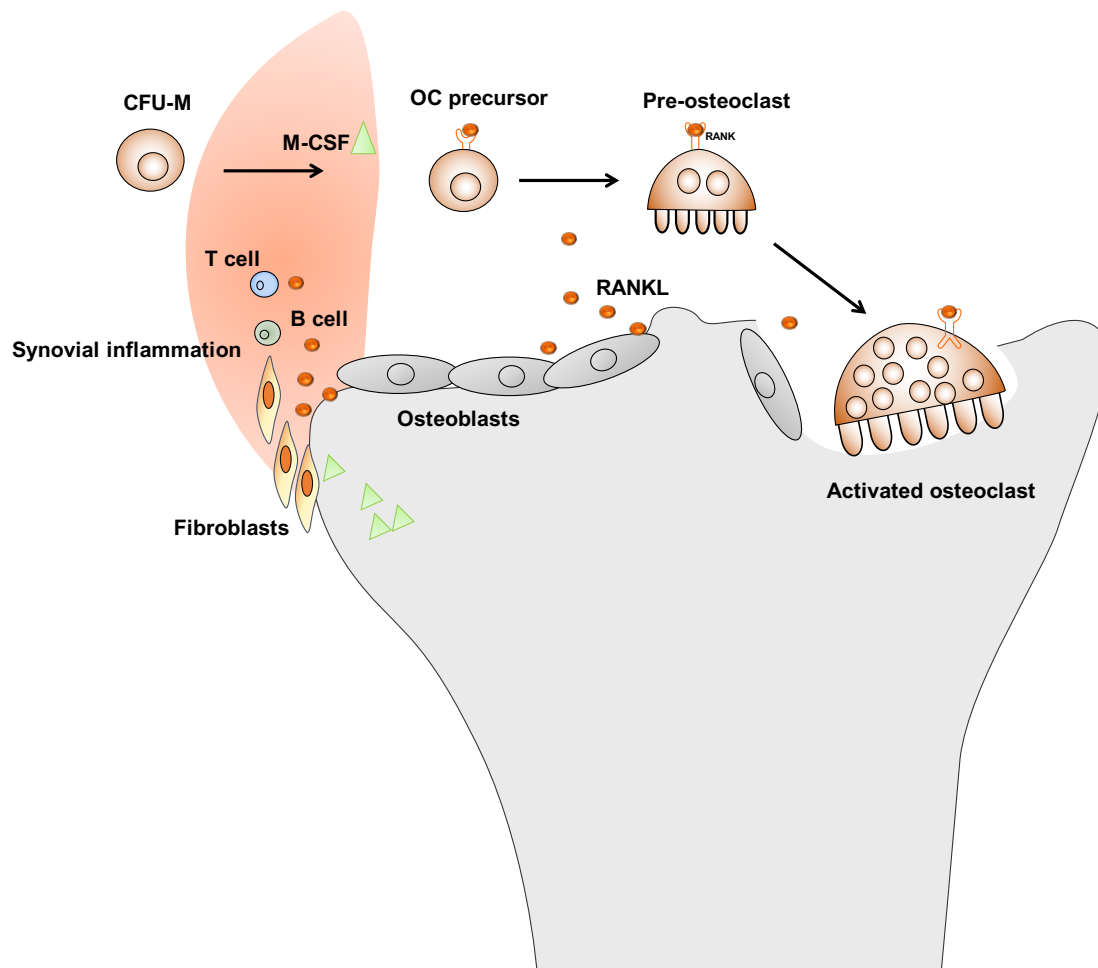

Figure 4: Inflammation induced bone loss

In the inflamed synovium, several proinflammatory mediators and activated cells such as fibroblasts and lymphocytes are present. These cells induce bone loss by directly releasing pro-osteoclastogenic cytokines stimulating cells increasing the differentiation OC precursor cells to into OCs thereby damaging bone.

Autocrine and paracrine signalling in OC development and functioning capacity has been implicated in the normal and pathological bone remodelling. One of the key mediator of the autocrine signalling in osteoclastogenesis is IL-8, which is regulated by RANKL and other pro-inflammatory cytokines involved are IL-6, TNF and IL-1 (192, 193). Though other cytokines can also be secreted by OCs, the magnitude of the IL-8 production is very high in the OC culture supernatants (193). Moreover, inhibiting IL-8 or the IL-8 receptors (CXCR1/2) during the OC development significantly reduced the OC formation (192).

Interestingly, circulating levels of the IL-8 is associated to the osteolysis in breast cancer metastasis patients (194). These findings on IL-8 suggest that it is an important autocrine factor which is essential for the OC development and functioning linked to pathological bone loss.

In addition to the RANK signalling, co-stimulatory pathways play an important role in osteoclastogenesis (195). Co-stimulatory molecules such as osteoclasts associated receptors (OSCAR) and paired Ig-like receptor-A(PIR-A) transmit their signal through with the FcγR chain associated adaptors (190). On the other hand, TREM2 (Triggering receptor expressed on myeloid cells) and MD-L1 (Myeloid Dap-12 associated lectin) are the other co-stimulatory molecules that are associated with DAP-12 (DNAX associated protein 12D size) (195). The importance of the co-stimulatory molecules has been characterized either by blocking these receptors or loss of function of their genes which impairs osteoclastogenesis (195).

1.4.3.3 The link between the bone and the immune system

To gain insight on how the immune system orchestrates bone formation and resorption, we need to understand the interplay between the bone and the immune system, which led to coining of the term 'osteoimmunology'(196). Immune cells and bone cells are known to exchange signals in the form of cytokines and receptor signalling. Osteoclastogenesis is one of the key event that is dis-regulated in the autoimmunity and this leads to increase in the bone erosion. Several studies on the T and B-cells have increased our understanding on the signals that arise from the adaptive immune systems that contribute to the bone remodelling. For instances, T cells can promote and inhibit osteoclastogensis. Activated T helper cells are known to promote bone destruction by directly by expressing RANKL in RA (197) and they can secrete cytokines like IL-17 which is known to indirectly augment osteoclastogensis (198). On the contrary, Foxp3 expressing T regulatory cells can inhibit the osteoclastogensis by secreting the IL-10, IL-6 and IFNgamma(199).

OCs are considered as the innate immune cells due to their capacity to express immunoreceptors, present antigens, sense the TLR mediated immunity (200). Activated B-cells are known to secrete pro-osteoclastogentic factors and they can support the development and activation of OCs (201). Synovial tissues and the SF is an important source of RANKL producing Fc-receptor-like-4(FcRL4) B-cells and activated T-cells contributing to the bone destruction in RA (197, 202). Interestingly, presence of autoantibodies against the citrullinated and carbymaleted targets are linked to the severe radiological progression

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suggesting that B cells can regulate osteoclastogensis (203) (42). Altogether these studies demonstrate the role of lymphocytes in regulating OCs both in the pre-clinical phase and the clinical phase of RA.

Prevention of bone destruction is an important aspect of the treatments for the RA patients. To further understand the link between the immune system and the bone, the therapies developed to target adaptive immunity can have direct and indirect effect in protecting the bone. Rituximab targets CD20 positive B cell and depletes those in RA patients, known as effective in treating the seropositive RA patients (204). Rituximab has been reported to abrogate the joint destruction indirectly by decreasing the synovial RANKL expression and the OC precursors population (205). Clinical studies with abatacept (CTL4 Ig) have reported that they are efficient in controlling the symptoms of RA and importantly a bone sparring effect (206). In relation to the clinical studies with abatacept, *in-vitro* studies using CTLA4 directly on the monocyte derived OCs abrogates the OC formation (207). These findings indicate a major link between the immune system and the bone.

2 AIM

The overall aim of my thesis is to investigate the pathogenic role of ACPA with a focus on bone destruction and pain induction. Specific aims are

1. To investigate the capacity of ACPAs to induce osteoclastogenesis
2. To investigate the capacity of ACPAs to induce pain-like behaviour *in-vivo*
3. To investigate the role of citrullination in OC development
4. To investigate the role of DCs transdifferentiation in OCs

3 MATERIALS AND METHODS

3.1 Patients

Informed consent was obtained from the RA patients at the Rheumatology Clinic at Karolinska Unviersity Hospital, Stockholm, Sweden. ACPAs were purified from Synovial Fluid (SF)(n=26) and Plasma (n= 38) from anti-CCP2 antibody positive RA patients fulfilling the American college of the Rheumatology (ACR)/ European League Against Rheumatism (EULAR) criteria for RA. For OC assays, blood was obtained from (n=6) ACPA positive RA patients. The regional ethics committee at Karolinska Institutet approved the study.

3.2 ACPA production (Paper I, II and III)

Isolation of the polyclonal ACPAs was performed using initial purification of the total IgG from the SF and Plasma of RA patients followed by ACPA IgG affinity purification on CCP2 columns as described previously (208). Non-ACPA IgG fraction (FT) is used as the control for the all experiments involving polyclonal ACPAs. Further, SF and PB ACPAs were characterized for peptide epitope recognition using a multiplex chip-based assay (Paper I).

Monoclonal ACPAs and control monoclonal antibody were generated using single B-cell or plasma cell isolated from RA patient SF. Followed by the isolation, immunoglobulins were cloned and expressed in human kidney embryonic fibroblasts (Gibco Invitrogen) or expi293 cells (Thermo Fisher scientific). The expressed antibodies were purified using protein G isolation column. Antibody reactivity was characterized by the reactivity to the citrullinated epitopes using multiplex microarray or ELISA. All of the antibody preparations were endotoxin free.

3.3 Osteoclasts cultures and Bone resorption analysis (All papers)

To study the effect of autoantibodies on osteoclastogensis, we first isolated monocytes from healthy blood donor buffy coats or from the peripheral blood (PB) of patients with ACPA-positive RA patients, by ficoll separation followed by positive selection with anti-CD14 microbeads. Either MΦs or iDC were generated form these monocytes. MΦ were generated by 3 days M-CSF stimulation of monocytes and further developed into OCs in the presence of M-CSF and RANKL. iDC were generated 6 days GM-CSF and IL-4 stimulation of monocytes and further developed into OCs in the presence of M-CSF and RANKL. Additionally, CD1c+ iDC were isolated from healthy donor buffy coats, using a CD1c$^+$ DC isolation kit (Miltenyi Biotec Norden AB, Lund, Sweden) and further developed into OCs using in the presence of M-CSF and RANKL. For studies of osteclastogenesis in mice,

CD11b$^+$ bone marrow cells were isolated using CD11b$^+$ isolation kit (Miltenyi Biotec Norden AB, Lund, Sweden) and further developed into OCs in presence of murine M-CSF and RANKL. In both human and mice experiments, OCs were identified by light microscope as tartrate-resistant acid phosphatase (TRAP) positive cells with at least 3 nuclei.
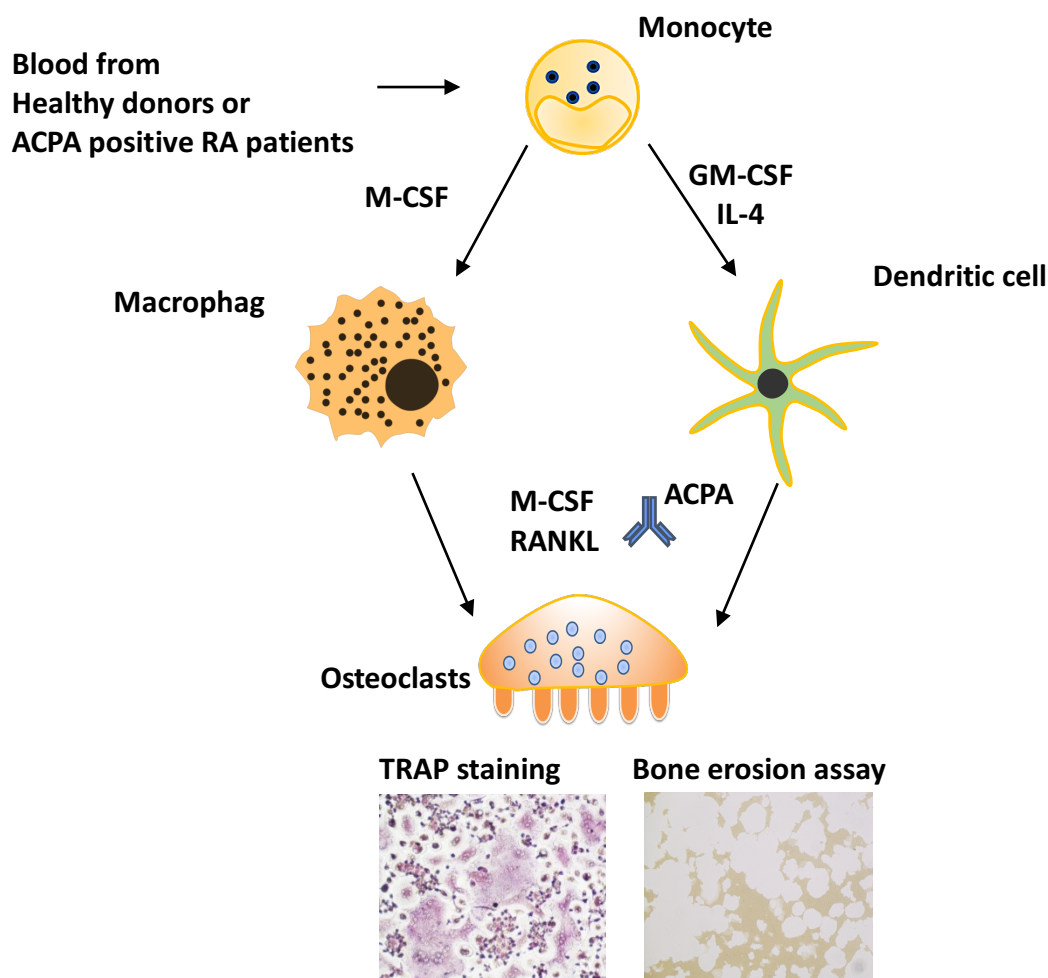

Figure 5: Schematic representation of OC development from peripheral blood monocytes derived MΦ and DC as precursors in the presence of ACPA.

To study the function of the OCs, we used an *in-vitro* model of bone erosion using an artificial bone surface, coated with inorganic crystalline calcium phosphate that mimics the physiological bone (Osteassay surface, catalogue number CLS 3988, Corning, NY, USA). MΦs and iDCs obtained were developed into OCs (as describe above) on these artificial bone surfaces. At the end of the culture OCs were removed and erosion zones were visualized under light microscope. Erosion area was estimated as percent of total area by NIS-elements basic research (Nikon, Amsterdam, Netherlands)

Cell culture supernatants from the TRAP assay were analysed for the cytokines using cytometric bead array (CBA).

3.4 Mass spectrometry (Paper III)

Mass spectrometry (MS) was used to study the proteome difference between MΦ- and iDC-derived OCs and to identify the citrullinated proteins. Whole cell lysates from OCs generated from MΦ- and iDC-precursors were used for the analysis. Protein concentrations were determined followed by trypsin digestion. Samples were analysed using Easy-nLC chromatography system directly coupled linked online to a Q Exactive mass spectrometer. Principal component analysis (PCA) was performed on the resulting proteome list after applying 1% false discovery rate (FDR). Label-free method was used to identify citrullinated proteins in our samples based on the 0.98 Da mass difference between citrulline and arginine detected in LC-MS/MS analysis. To avoid misidentification of the peptides, we visually inspected and validated the spectral fragmentation pattern of the citrullinated peptides.

3.5 PAD activity (Paper I and III)

PAD enzyme activity in the cell lysates was measured using the commercially available ELISA kit measuring the amount of arginine that have been deiminated. PAD enzyme activity was determined by correlation against the control recombinant human PAD4 enzyme activity.

3.6 Immunohistochemistry and Immunofluorescence (Paper I, II and III)

PAD enzymes and citrullinated protein expression was analysed using both permeabilized and non-permeabilized staining procedure of OCs cultured on glass slides and mice joint tissue sections. Commercially available and in-house engineered monoclonal antibodies were used and visualized with fluorescent dyes or 3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB) using confocal microscope and light microscope respectively.

3.7 Animal Experiments (Paper I and II)

Experiments involving mice were approved by local ethical committee for animal experiments Stockholm, Sweden. Adult male Balb/c mice were used to investigate the *in-vivo* effect of ACPAs. Animals were injected intravenously with either ACPA IgGs or murinised monoclonal ACPAs and respective control IgGs. To assess the mechanical sensitivity, mice were acclimatized on top of mesh like surface and optiHair filaments were applied under the paw until buckling of the hair filament occurred. Positive response was determined using the spontaneity of withdrawal within 2-3 seconds and 50 % withdrawal was calculated using

Dixon up-down method. At the end of the experiment mice were euthanized and tibia from left hind leg was used for bone structure was analyzed using a sky scan micro-CT (microcomputer tomography). Trabecular bone in tibia which is located 644μm from proximal growth plate and extending 100,5μm was assessed for bone mineral density (BMD) and 3D analysis. Volume of cortical bone in tibia measuring 617μm in length, located in the distal tibia was analyzed for tissue mineral density(TMD) and 3D analysis, by CT Analyser software (version 1.14.4.1; Bruker).

3.8   Flow cytometry (Paper III and IV)

MΦs and DCs were analyzed for the phenotypic markers using commercially available antibodies conjugated with flourochromes and the data was analyzed using Flow Jo software.

3.9   Statistical analysis (All papers)

Kruskal-Wallis test followed by Dunn's multiple comparison and one-way ANOVA followed by Holm-Sidaks post hoc test were used for non-parametric and parametric comparisons of three or more groups. Two-way analysis of variance followed by Bonferroni post hoc test was used for repeated measurements comparing changes over time. Graph Pad prism 6 software was used for the all the statistical analysis. Probability (P) values less than 0.05 were considered significant.

4 RESULTS AND DISCUSSION

Bone destruction and pain are important clinical features in RA. Pain in the joints without clinical inflammation, known as arthralgia, in combination with ACPA positivity has been shown to be highly predictive for RA development (209, 210). ACPAs are present in the blood long before the onset of RA (41) and are a major risk factor for developing bone loss even in the absence of clinical signs of inflammation (152, 203). We aimed to investigate the role mechanistic link between the ACPA positivity, bone destruction and pain.

4.1 Polyclonal ACPAs enhance osteoclastogenesis and bone resorption in vitro

To test ACPAs effects on OCs, we first used polyclonal affinity purified ACPA derived from the peripheral blood and synovial fluid of RA patients. As expected, polyclonal ACPA showed broad reactivity against several citrullinated targets including several citrullinated epitopes in vimentin, enolase and fibrinogen. Polyclonal ACPAs but not control non-ACPA IgGs, enhanced the number of MΦ-derived OCs and their bone resorptive capacity. The effect was equally potent from MΦ -derived OCs originating from both healthy individuals and ACPA-positive RA patients. For OCs originating from ACPA-positive RA patients, polyclonal ACPAs increased the OC numbers by a fold of 1.8±0.6 (mean±SEM) and bone resorption by a fold of 2.3±0.7 (mean±SEM) when compared to non-ACPA IgGs (p<.0.05, figure 6A and B). We further investigated if the effect of polyclonal ACPA was dependent on OC cell precursors by testing ACPAs effect on the OC transdifferentaition of both in vitro generated healthy donor monocytes-derived iDC and healthy donors blood isolated CD1c+ iDC.

We next wanted to investigate if ACPA effect is dependent on the cellular type of OC precursors. As beside MΦ, iDC have been previously described as potent OC precursors we investigated the capacity of polyclonal ACPA to enhance osteoclastogenesis from in vitro developed iDC originating from healthy donors. Similar to MΦ precursors, ACPA IgGs were able to promote osteoclastogenesis from iDC precursors with a significant increase in both OC numbers (a fold increase of 2.3±0.9, p<0.05, figure 7A) and bone resorption area (a fold increase of 2.6±0.9, p<0.05, figure 7B) compared to control IgGs. Principal component analysis on the cell proteome confirmed that the iDC and MΦ precursors have distinct proteomic signatures while OC derived from either of these precursors have indistinguishable proteomic signatures (Paper III). To further test the reproducibility of the model and to mimic a putative in vivo situation, circulating myeloid DC precursors, CD1c+ DCs, were isolated

from blood of healthy individuals and stimulated with ACPAs. We were able to demonstrate for the first time that also circulating iDC are capable of OC transdifferentiation and this is enhanced by ACPA in a similar manner as for MΦ and in vitro developed iDC (Paper III).
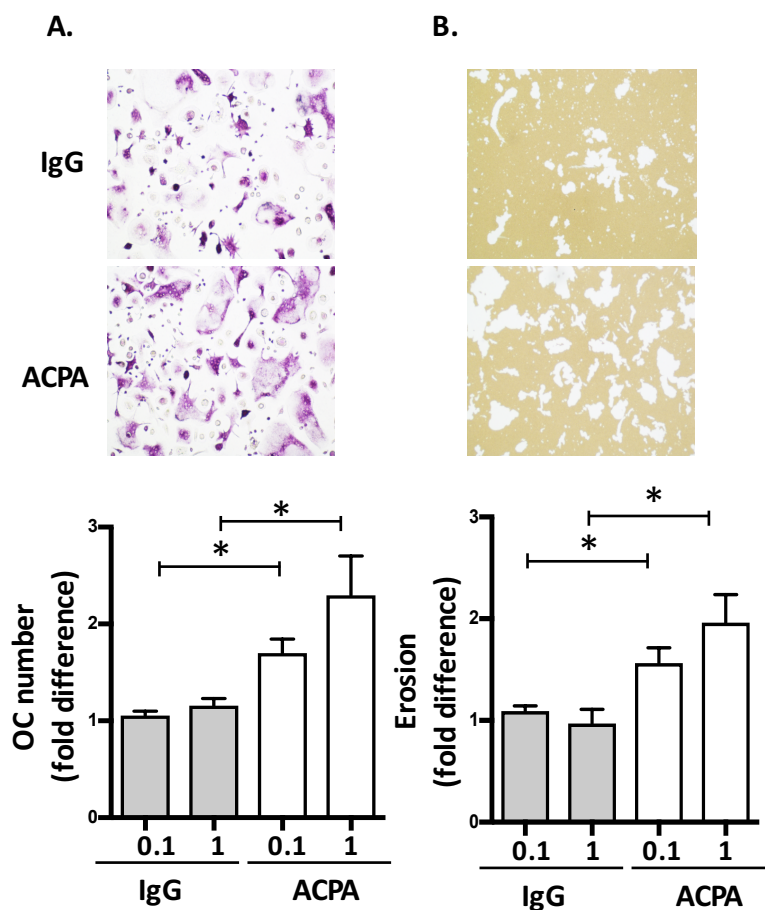

Figure 6: ACPAs promote osteoclastogenesis and bone resorption

MΦ-derived OCs generated from ACPA positive RA patient B. (A) TRAP positive OCs treated with PB-derived polyclonal ACPAs or control IgGs. (B) Bone resorption by OCs generated on the calcium phosphate surface. Graphs represent the fold difference in OC number and erosion area in the presence of 0.1 and 1 µg/ml of IgGs or ACPAs. *p<0.05.
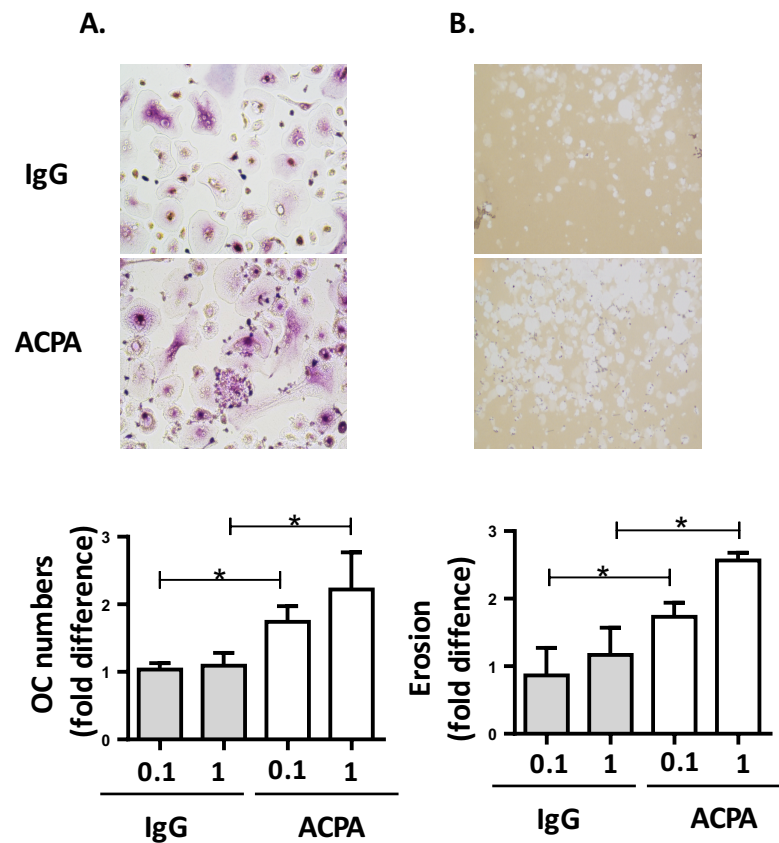
Figure 7: ACPA increases iDC transdifferentiation into OCs

iDC-derived OCs generated from peripheral blood of RA patients in the presence of control IgGs or ACPAs. The number of OCs analyzed using (A) TRAP staining (100X magnification) and (B) calcium phosphate bone resorption assays. Images were obtained using light microscopy and graphs represent the fold-difference (normalized using untreated M-CSF/RANKL controls) in OC numbers (TRAP-positive cells with ≥3 nuclei) and in resorption areas (n=3 independent experiments).

Taken together our findings show that polyclonal ACPA with broad specificities are able to enhance osteoclastogenesis and bone resorption independent of what type of OC precursors are used (either MΦ or iDC derived, either healthy donors or RA patients derived). This is in agreement with others studies showing that also more restricted polyclonal ACPAs such as antibodies against MCV are able to promote MΦ derived osteoclastogenesis (102). However, due to the large number of cit specificites recognized by the polyclonal ACPA it is impossible to predict which individual or combination of specificities is responsible for this effect. Based on our iDCs findings it is tempting to speculate on a role for these cells in RA-associated bone loss. Increased frequency of DCs are found in SF of RA patients with a decrease in DC levels in circulation (211). It appears that DC migrated into the joint might have the capacity

to switch from their conventional function of antigen presentation to undergo transdifferentiation in to OCs in the presence of ACPAs. In line with this hypothesis it has been shown that both immature and more mature DCs express RANK, an essential molecule for OC differentiation and maturation (212, 213).

4.2   Some but not all monoclonal ACPAs enhance osteoclastogenesis

In order to further investigate the relevance of epitope recognition and antibody traits for the above described effect we went on to test the effect of of monoclonal ACPAs obtained by single B cell cloning from the SF of RA patients. Interestingly, while monoclonal ACPAs had a more limited reactivity they still showed significant cross-reactivity with different patterns: 1325:01B09 (CCP1, Cit-Fibβ_{36-52} and Cit-Fib$_{563-583}$) and 1325:04C03 (CCP1, Cit-Vim$_{60-75}$ and Cit-vim$_{2-17}$) (see figure 8). Importantly none of the ACPA monoclonal showed any kind of reactivity towards native non-modified targets. This characteristic of monoclonal ACPAs have been now reported in others studies from our laboratory (101). Out of four tested monoclonal ACPAs, two were able to enhance osteoclastogenesis and bone resorption while two others failed to do this (Paper I). Importantly again neither E02, an irrelevant antibody directed against an epitope of the tetanus protein, nor a RF (un-published, see figure 9), both isolated using the same methodology as for the monoclonal ACPAs had any effect on OCs. This data taken together show that antibody traits and recognition patterns are important. However due to the above described cross-reactivity it is difficult to speculate on the exact specificity or specifcities that are and those that are not related to the ACPA osteoclastogenetic effect. Notably, ACPAs that induced osteoclastogenesis reacted with the immunodominant cit-epitopes of vimentin (Vim$_{60-75}$) and enolase (CEP1) along with other epitopes, while ACPAs that failed to induce OC mainly reacted with other epitopes such as those derived from fibrinogen.

We than wanted to investigate if the observed in vitro effect has relevance in vivo. To do this we injected monoclonal ACPAs into Balb/c mice and evaluated bone loss by micro-CT. Confirming the in vitro observations ACPA injection in mice induced significant trabecular bone loss and some occasional cortical erosions (see figure 13)
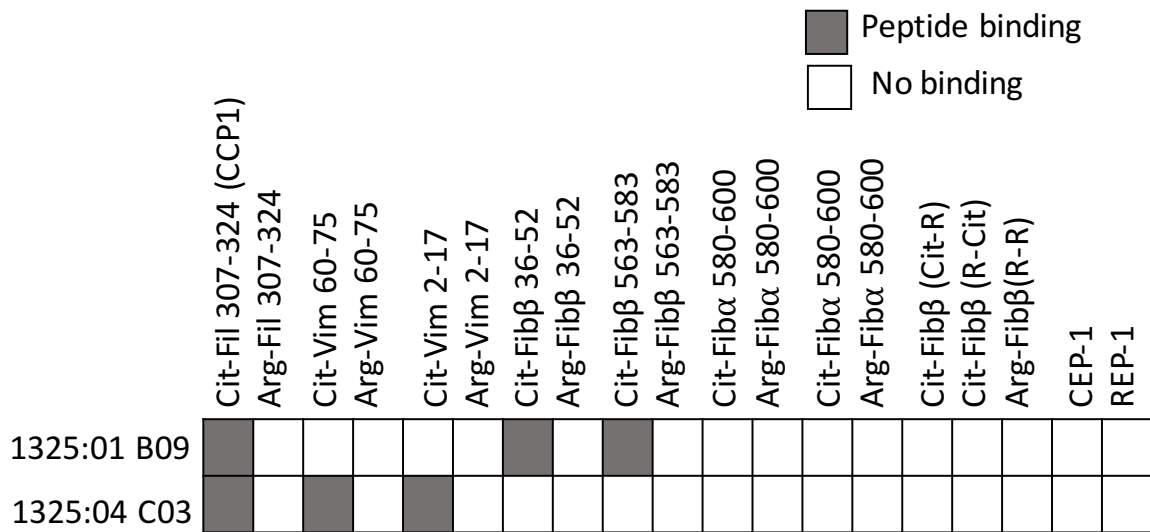
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	Cit-Fil 307-324 (CCP1)	Arg-Fil 307-324	Cit-Vim 60-75	Arg-Vim 60-75	Cit-Vim 2-17	Arg-Vim 2-17	Cit-Fibβ 36-52	Arg-Fibβ 36-52	Cit-Fibβ 563-583	Arg-Fibβ 563-583	Cit-Fibα 580-600	Arg-Fibα 580-600	Cit-Fibα 580-600	Arg-Fibα 580-600	Cit-Fibβ (Cit-R)	Cit-Fibβ (R-Cit)	Arg-Fibβ(R-R)	CEP-1	REP-1
1325:01 B09	■						■		■										
1325:04 C03	■		■		■														

Legend: ■ = Peptide binding, □ = No binding

Figure 8: Specificity pattern of monoclonal ACPAs

ACPA reactivity pattern as analyzed on a multiplex microarray of RA associated peptides (214). Peptide binding is indicated by grey color.
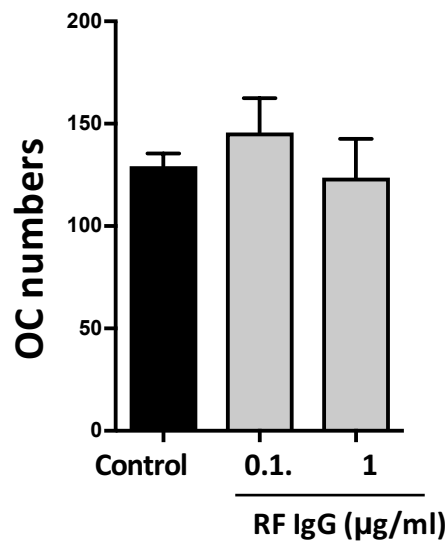
Figure 9: Rheumatoid Factor IgG has no influence on osteoclastogenesis

MФ-derived OCs generated from healthy donor buffy coat. (A) TRAP positive OCs treated with RF IgG and bar graph represents the OC numbers.

4.3 ACPAs effects on OC are dependent on protein citrullination

As ACPA but no other control antibodies were able to mediate osteoclastogenesis, we hypothesized that protein citrullination in OCs might be a previously unrecognized regulatory mechanism for osteoclastgenesis. We first tested if ACPA are able to bind to OC being able to demonstrate that ACPA but not the non-ACPA antibodies are able to bind targets

expressed on the surface of non-permeablised OC suggesting that citrullinated target on the surface of OCs might be the initiating step in ACPA-mediated osteoclastogenesis (Paper I). A previous report had shown that cit-vimentin can be identified by label free MS in MΦ derived OC (102). Using a similar approach, we also investigated presence of cit targes in iDC derived OCs and showed that three citrullinated beta-actin peptides were identified in iDC precursors, while the mature iDC derived OCs expressed an increased number of citrullinated peptides, eight being beta-actin peptides and six vimentin peptides (Table A in figure 10). To confirm this with than studied if ACPAs can bind to either actin or vimentin on the surface of OC. Indeed, ACPA co-localised with antibodies detecting either vimentin or actin (figure 10B). while these finding point to a potential role of cit actin and vimentin, it is important to note that other cit targets derived from low abundant proteins might have been missed in our MS screening.

A.

Peptide Sequence	Amino acid	iDC	OC
Beta-actin			
AGFAGDDAP(Cit)AVFPSIVGRPR	19-39	28	26
AVFPSIVG(Cit)PR	29-39		10
(Cit)GILTLK	62-68		46
IWHHTFYNEL(Cit)VAPEEHPVLLTEAPLNPK	85-113	41	46
ILTE(Cit)GYSFTTTAER	192-206		41
GYSFTTTAE(Cit)EIVR	197-210		44
DLYANTVLSGGTTMYPGIAD(Cit)MQK	292-315		11
QEYDESGPSIVH(Cit)K	360-373	31	37
Vimentin			
SLYASSPGGVYAT(Cit)SSAVR	51-69		68
NT(Cit)TNEKVELQELNDR	98-113		124
DV(Cit)QQYESVAAK	271-282		44
FADLSEAAN(Cit)NNDALR	295-310		42
KLLEGEES(Cit)ISLPLPNFSSLNLR	402-424		42
LLEGEES(Cit)ISLPLPNFSSLNLR	403-424		39

B.

Figure 10: Identification and validation of citrullinated proteins in iDCs and OCs

(A.) Table representing citrullinated peptides identified in iDCs and OCs. Numbers indicate the highest mascot score obtained for each peptide and blank spaces indicating undetectable citrullinated peptide. (B) OCs double stained for vimentin or actin respectively in green, together with monoclonal 1325:04 C03 ACPAs in red with nuclear staining using DAPI in blue. Representative confocal microscope images analyzed using image J for co-localization analysis in grey and r is the correlation coefficient.

Being able to identify citrullinated targets in OC, we than investigated the expression patterns for the enzymes that are responsible for citrullination of protein, namely peptidyl arginine deiminases, focusing on PAD2 and PAD4 that have been previously identified in monocytes, MΦs and the inflamed rheumatoid tissue (68, 215). Both enzymes were present in OC precursors with an increase in PAD2 and decrease in PAD4 expression in mature OCs (Paper I and III). Further, significant PAD activity was detected in both OC precursors and more mature OCs (figure 10A). Presence of citrullinated targets and active PAD enzymes in OCs together with the original observation that ACPA promote OC differentiation and maturation prompt us to investigate if PAD enzymes are involved in ACPAs effects. To this end, we tested the effect of a pan PAD inhibitor, Cl-amidine, on OC differentiation in the presence and absence of ACPA. To our surprise Cl-amidine inhibited OC differentiation even in the absence of ACPA and despite high RANKL concentration, suggesting that PAD activity is essential for the physiological development of OC (figure 10B). Interestingly, Cl-amidine did not affect the functions of other cells such as synovial derived fibroblasts (Paper I).

Taken together these findings suggest that the OCs but no other cells are dependent on citrullination and PAD activity for fulfilling their physiological role. Such a tentative unique feature of OCs might explain the presence of citrullinated proteins on the OCs during their normal differentiation, in contrast to most other cells that express citrullinated proteins only in an inflammatory milieu (216). This dependency on PADs and citrullination for normal OC differentiation might therefore be linked to preferential targeting of OCs by ACPAs in a non-inflammatory context. This scenario is further supported by the observation that Cl-amidine was able to inhibit ACPA-induced osteoclastogensis as well. As such high Cl-amidine doses completely block ACPA-induced ostelcatogenesis, while lower doses of Cl-amidine (0.2μM for MΦ-derived osteoclastogenesis and 2μM for iDC derived osteoclastogenesis) blocked only ACPA-induced osteclastognesis but not basal (RANKL dependent) osteoclastogenesis (figure 11 C and D).
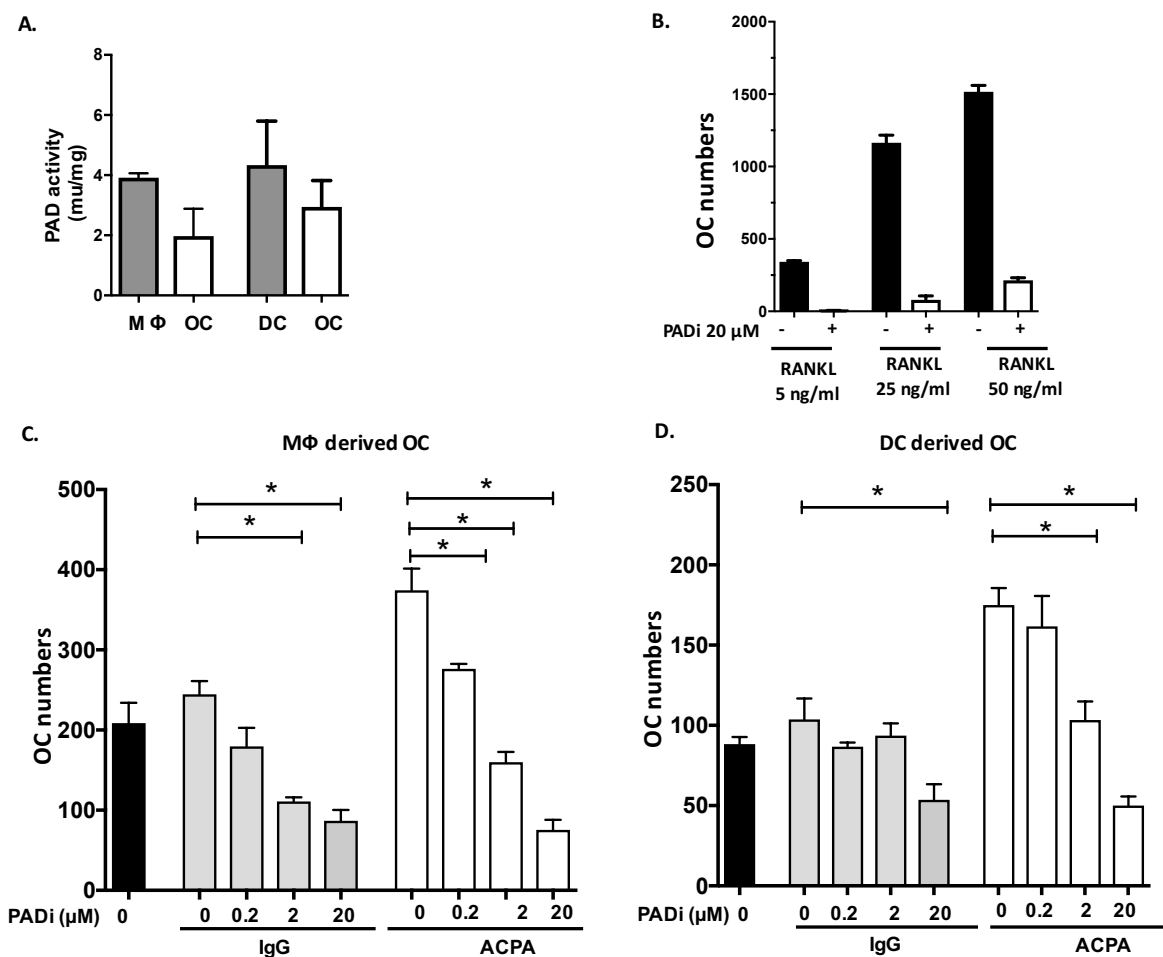
Figure 11: Citrullination and PAD enzymes are essential for OC differentiation and ACPA-mediated osteoclastogenesis. (A) PAD activity was measured in MΦ- and DC-derived OC cell lysates. Bar graph represents the PAD activity expressed in mu per mg (n≥3 independent donors) (B) Osteoclastogenesis with 5, 25 and 50 ng/ml of RANKL in the presence of 10 ng/ml M-CSF with and without PAD inhibition (20μM). (C and D) ACPA-effect on MΦ- and DC- derived osteoclastogenesis in the presence of PAD inhibitor. Bar graphs illustrating the fold difference in OC numbers based on TRAP staining (n≥3 independent experiments). *p<0.05

4.4 ACPA activate osteoclstogenesis through and IL-8 dependent mechanisms

To further explore potential cellular mechanisms responsible for mediating ACPAs effect we measured a panel of cytokines in OC culture supernatants. Of several investigated mediators, IL-8 concentrations were increased in the presence of ACPA but not control non-ACPA IgG (figure 12 A and B). It has been previously shown that IL-8 is able to promote osteoclastogenesis by an autocrine mechanism and we show here evidence that this mechanism might be involved in ACPA induced osteoclastogensis. Confirming this, IL-8

blocking by monoclonal neutralising antibody abolished the ACPA-mediated osteoclastogenesis (figure 12 C). We then proceed to investigate if IL-8 mediates ACPAs effect in in vivo model. To this end we injected ACPA in mice in the presence or absence of reparixin a CXCR1/2 inhibitor blocking the effect of CXCL1 and 2, the mouse homologues for IL-8. Confirming the in vitro result reparixin was able to abolish ACPA-induced trabecular bone loss as measured by micro CT (see figure 13)
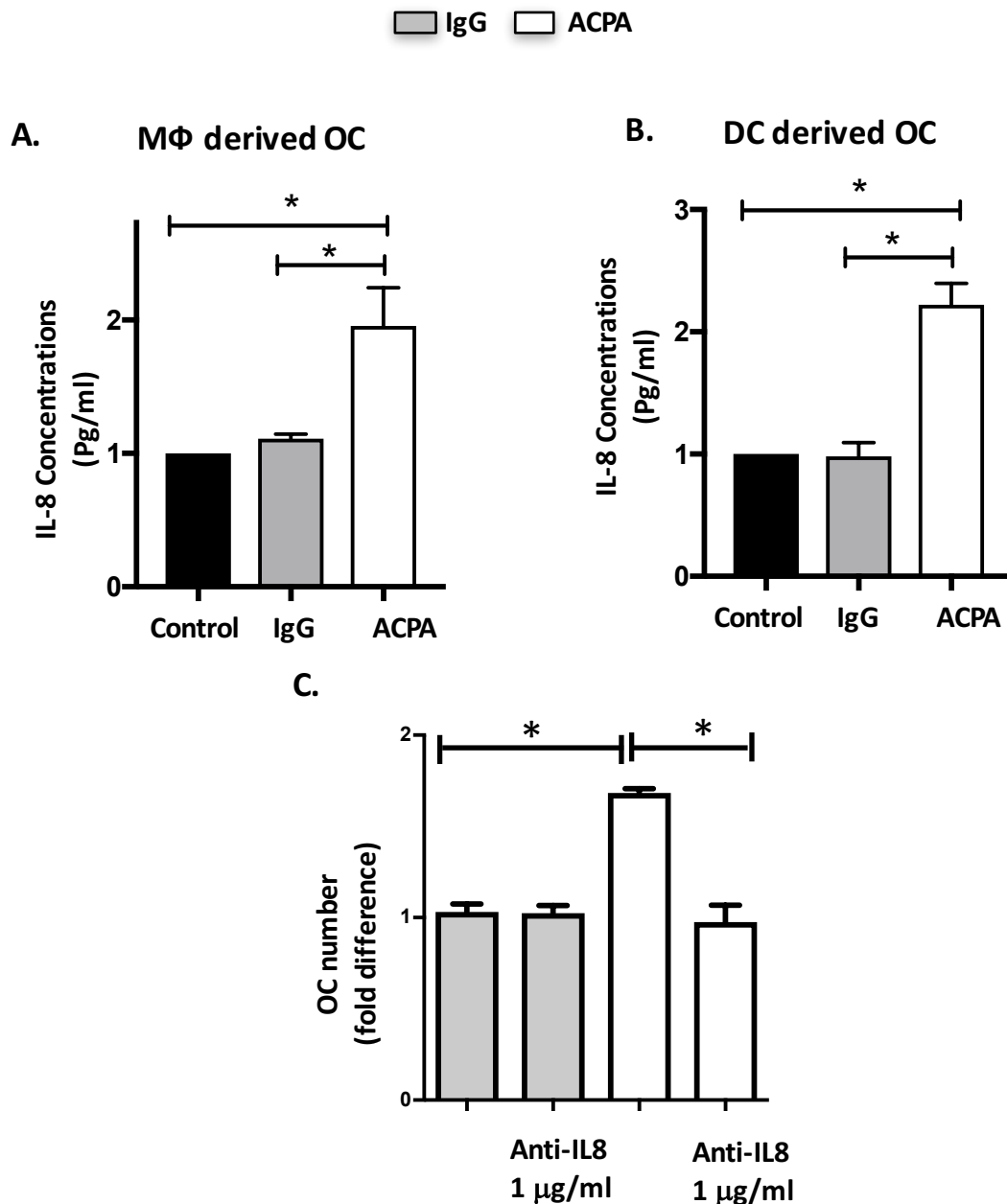
Figure 12: ACPA increases IL-8 levels during osteoclastogenesis

(A and B) Graphs representing the IL-8 levels measured in supernatants from IgG or ACPA treated MΦ and DC derived OCs. (C) OC culture in the presence of IgGs or ACPAs with and

without IL-8 neutralizing antibody (1 µg/ml) (C) Graphs illustrating the fold difference in OC numbers monitored using TRAP staining. *p<0.05.
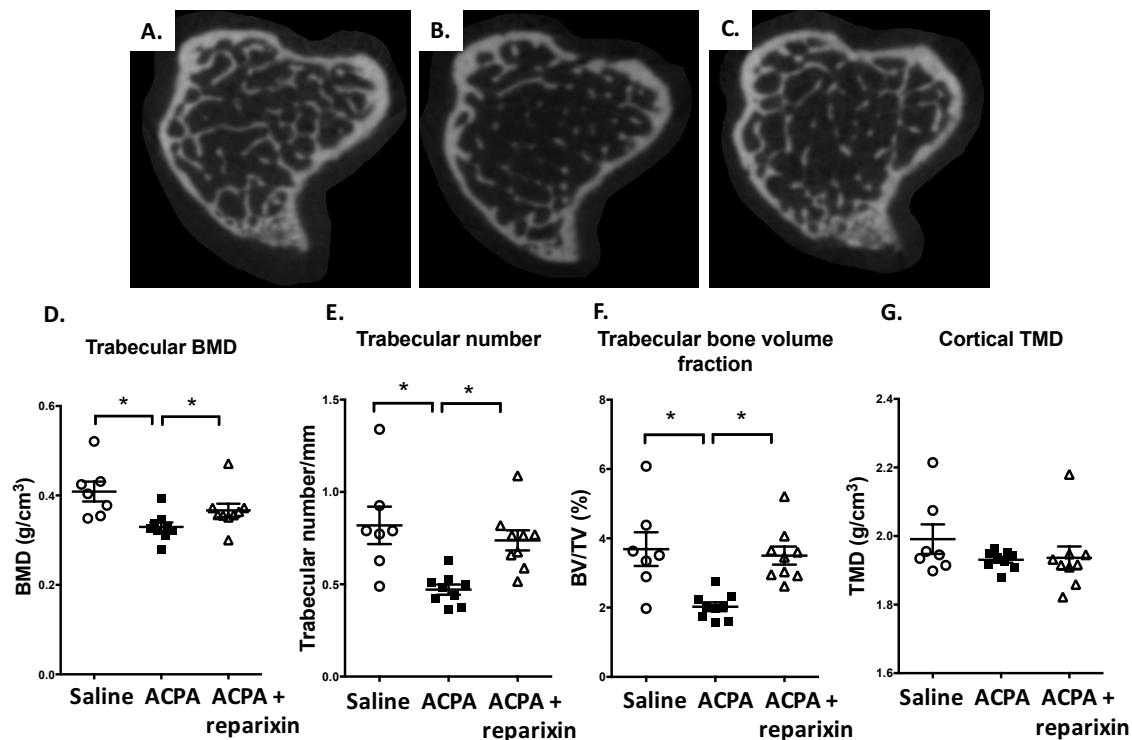
Figure 13: Systemic ACPA-induced bone loss is neutralized by reparixin targeting CXCR1/2. A) Micro-CT images in 2D representing changes in tibial bones of mice injected with saline, (B) ACPA and (C) ACPA followed by reparixin treatment. Graphs representing (D) trabecular bone mineral density (BMD), (E) trabecular number, (F) trabecular bone volume (BV/TV) and (G) cortical trabecular mineral density (TMD). *p<0.05.

4.5 ACPAs induce pain-like behavior in mice

As ACPAs and bone loss are present in individuals at risk for developing RA presenting with arthralgia, we aimed to investigate a potential connection between ACPA, bone loss and pain. To do this we used a mechanical sensitivity model developed to study pain in mice. Mice were administered i.v. with either human polyclonal ACPA, control IgG (FT) or saline and the mechanical sensitivity was followed for 7 or 28 days. ACPAs induced pain-like behaviour in mice with significantly lower tactile threshold at all time points from day 1 as compared to non-ACPA IgG and saline treated mice (figure 14A and C). As ACPAs stimulate OC to produce IL-8 and IL-8 is a known pain-inducer molecule we hypothesize that IL-8 might be the link between ACPA-activated OCs and pain. To test this CXCL1/2 is known to induce pain-like behaviour in mice by binding CXCR2 expressed on the peripheral and nociceptive neurons (217).

We sought to determine the link between the bone loss and pain performing immunofluorescence stainings on the sub-chondral bone of mice using polyclonal ACPAs and anti-CD68 antibodies. Strikingly, ACPAs specifically co-localised with the CD68 positive MΦ, possibly OC precursors in the bone marrow with no affinity to synoviocytes, osteocytes, chondrocytes or endothelial cells (figure 14B). Furthermore, the double positive CD68/ACPA OC precursors were in close proximity with the CGRP positive sensory fibres in the bone marrow (Paper II), providing a putative link between OC-derived CXCL1/2 and sensory nerve stimulation. It is important to mention that *in-vitro* generated OCs from mice bone marrow cells, stimulated with ACPA, increased the CXCL1 production in joints and increased CXCL1 and 2 levels were observed in ankle joint extracts from mice injected with ACPAs (Paper II).
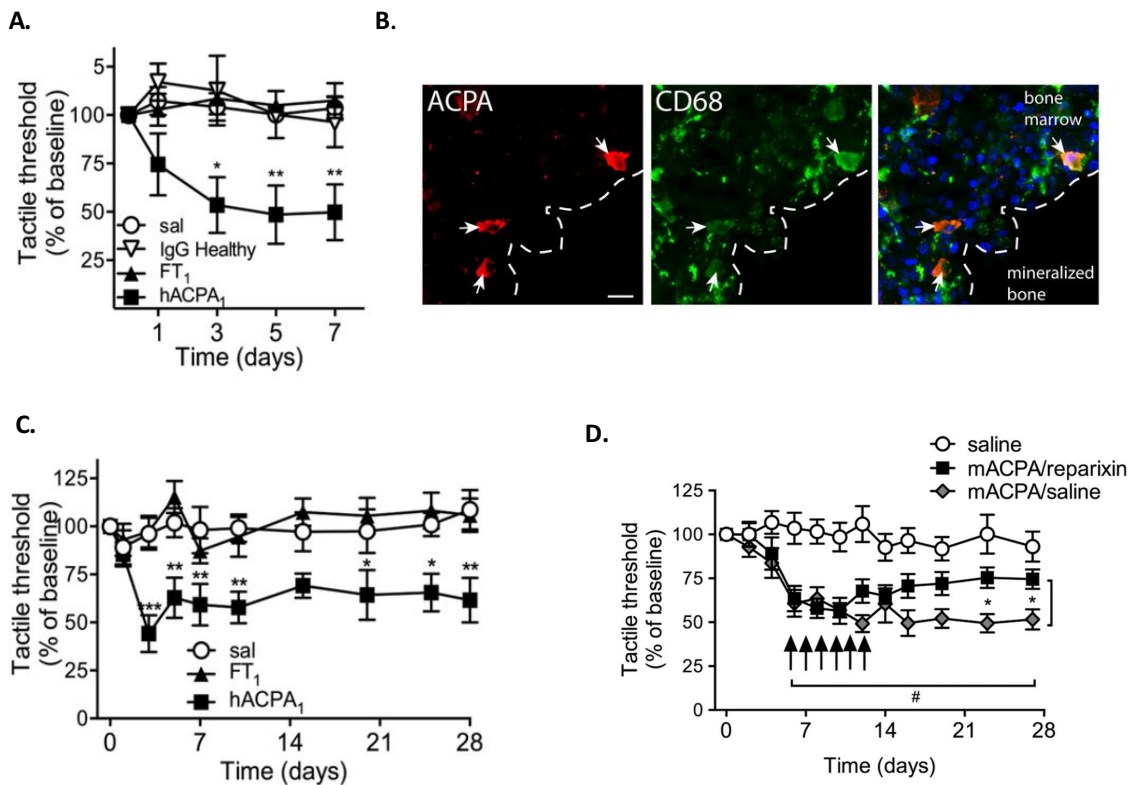
Figure 14: Mechanical sensitivity measured in mice after intravenous administration of human polyclonal ACPA and murinised monoclonal ACPA.

Saline, IgG from healthy donors, IgG from RA patients (FT) or polyclonal human ACPA was injected in equal amounts (1 mg per mice). A) Mechanical sensitivity was monitored for 7 days. (B) Microscopic images representing the sub-chondral mice bone marrow stained for OC precursors (CD68) in green and ACPA in red followed by the co-localization of ACPA with CD68 positive cells in white (indicated with white arrows). (C) Mechanical sensitivity

induced by ACPA monitored for 28 days with (D) sub-cutaneous injections of reparixin (30mg/kg/day) between day 6 to 12.

To mitigate the pain induction mediated through CXCL1/2, we injected mice with monoclonal ACPAs (clones D10 and B02) and treated with CXCR1/2 antagonist reparixin for six consecutive days. Importantly, reparixin treatment partially reversed the ACPA-induced mechanical sensitivity, demonstrating that the attenuation of pain is dependent on the ACPA-mediated chemokine release by OCs (figure 14D).

In summary, we demonstrate that ACPAs promote bone loss and pain like-behaviour by inducing IL-8 both in vivo and in vitro. We show that ACPAs might bind to OCs in bone marrow which are localized near the CGRP positive sensory neurons and that the blockade of CXCR1/2 reduces the ACPA-induced hypersensitivity. Blocking IL-8 can also inhibit osteoclastogenesis and prevents ACPA-induced bone loss.

However, it is important to point out that ACPA administration alone did not lead to arthritis development indicating that ACPA-dependent arthritis-development might need a second hit causing inflammation. Moreover, the identification of ACPA targets on OCs and precise downstream signalling pathways remain to be explored. It has been previously reported that the anti- mutated citrullinated vimentin (anti-MCV) (Fab)2 fragments, generated by pepsin digestion, induced an increase in OC numbers (102). In our study, we used Fab fragments of the monoclonal ACPAs D10 and B02, which induced an increase in osteoclastogenesis (Paper I). This indicates that the ACPAs binding on citrullinated antigens expressed on the OC surface, play an important role in enhanced OC maturation and activation.

ACPA immune complexes have been shown to induce TNF production in MΦs (218). MΦs and OCs express Fc receptors on the cell surface and depending on the antibody isotype these immune complexes may activate or inhibit osteoclastogenesis (219). Recently, it has been reported that the presence of ACPA together with RF, additively increased the association with bone erosion in RA patients while RF alone was not associated with erosion size (220, 221). Interestingly, in agreement with this we demonstrated that RF IgG alone did not influence the OC development in vitro. It is tempting to speculate that the ACPA and RF immune complexes would further augment the ACPA-induced OC leading to increased bone erosion in RA. Our findings suggest that the Fc and the antigen binding Fab fragments have an independent role in OC maturation, but additional studies are needed to understand the complex role of antibody-mediated osteoclastogenesis.

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The pain-like behaviour was inducible using both the human polyclonal- and murinised monoclonal ACPAs originating from RA patients, demonstrating that pain induction in mice is not due to adverse reaction against human protein. In in-vitro OC experiments monoclonal ACPA Fab fragments were efficient to induce osteoclastogensis and enhance IL-8 production, demonstrating the importance of antigen antibody interaction (Paper I). It would be interesting to use ACPA Fab fragments in the pain-behaviour model to identify the importance of pure antigen-antibody-mediated effect.

To study the direct effect of ACPA on sensory neurons, we further investigated the effect of ACPAs on the peripheral neurons in in vitro cultures. ACPA-treatment did not affect the calcium in flux/ signalling in neurons (Paper II) suggesting that OCs are identifiable key targets for the ACPA-mediated IL-8 release. ACPAs precede the onset of RA but not all individuals with ACPAs report pain in the joints. Retrospective epidemiological studies on ACPAs report that over the years an increased antibody epitope recognition and increased ACPA titres close to the clinical disease development (222), indicating that certain ACPAs might be responsible for pain induction. In future, our animal model could be used to address this interesting question by using ACPAs from those individuals that report pain and those that are ACPA positive without pain.

Taken together, this study provides novel mechanism for ACPA-induced bone loss and pain signifying the potential pathogenicity of these autoantibodies. ACPAs are known to precede the clinical signs of RA and several studies have confirmed the occurrence of bone loss in ACPA positive individuals at risk of developing RA (152, 203, 223). Our findings suggest that ACPAs are not only bystander antibodies but actively involved in the OC activation in RA. Therefore, alleviation of pain symptoms in ACPA positive individuals by IL-8 blocking may decrease the ACPA-mediated OC activation and thus be a potent treatment to prevent RA development.

4.6 Lactic acid influences the dendritic cells transdifferentiation in to osteoclasts

Synovial cell accumulation enhances the consumption of oxygen leading to hypoxia in the joints (224). Additionally, activated Th1 or Th17-type lymphocytes, inflammatory MΦs or activated DCs turn towards glycolytic energy production, which facilitate biosynthetic processes and cytokine production. Acidiosis is a phenomenon of decreased pH, occurring due to the metabolic shift associated with hypoxia and immune activation (225). In RA, elevated levels of lactic acid have been observed in the inflamed joints (226). DCs or their

progenitors are recruited into the inflamed joints and contribute to antigen presentation and the production of pro-inflammatory cytokines or, alternatively, DC can also transdifferentiate into OC (175, 227). We aimed to study role of acidosis in the differentiation switch between inflammatory or OC-prone DC subsets.

We have observed a cell concentration-dependent reprogramming of monocyte-derived DC differentiation that was influenced by the amount of lactic acid in the cell culture supernatant (paper IV). DCs developing in sparse cultures ($0.125x10^6$ and $0.5x10^6$ cells/ml) homogenously expressed CD1a whereas DCs from dense cultures ($2x\ 10^6$ cells/ml) expressed both CD1a and CD14 (figure 15A). DCs developing in sparse cell cultures had higher capacity to produce inflammatory cytokines, to induce Th1 polarization, and to migrate towards the lymphoid tissue chemokine CCL19. On the contrary, activated DCs originating from the dense cultures produced IL-10 but no inflammatory cytokines (Paper IV).

Next, we investigated whether the different DC types obtained from dense and sparse cultures could trans-differentiate into OCs in the presence of M-CSF and RANKL. Interestingly, despite their non-inflammatory phenotype, DCs from dense cultures were more prone to develop into OCs and possessed higher bone resorption capacity (figure 15 B and D). To understand the mechanism that promoted different DC phenotypes in dense and sparse cultures, we added day-2 supernatants from dense to sparse cultures. In response to this treatment a clear downregulation of the $CD1a^+CD14^-$ expression profile was detected, even when only the <3KD fraction of the supernatant was used, suggesting a non-protein type mediator (Paper IV). Previously, it has been reported that lactic acid, produced by cancer cells can inhibit the development of IL-12 producing $CD1a^+CD14^-$ DCs (228). Interestingly, we detected similar lactic acid concentrations in dense DC cultures as previously observed in the case of cultures tumor cells (Paper IV).

To understand the role of endogenous lactic acid in the autocrin regulation of DC development we pre-treated DC precursors with oxamic acid, an inhibitor of glycolysis, or provided galactose instead of glucose in the cell culture medium. The reduced glycolytic activity increased the IL-12 producing capacity in the developing cells but interfered with their ability to transdifferentiate into OCs, suggesting a key role for lactic acid in the differentiation plasticity of DCs (figure 15D). Furthermore, the capacity of DCs to develop into OCs correlated with their endogenous PAD activity and the level of protein citrullination, indicating that the DC-OC transition could also be regulated by protein citrullination. Moreover, DCs that turn towards the OC-prone lineage might also be sensitized to the ACPA-mediated boost in OC development (Paper III).
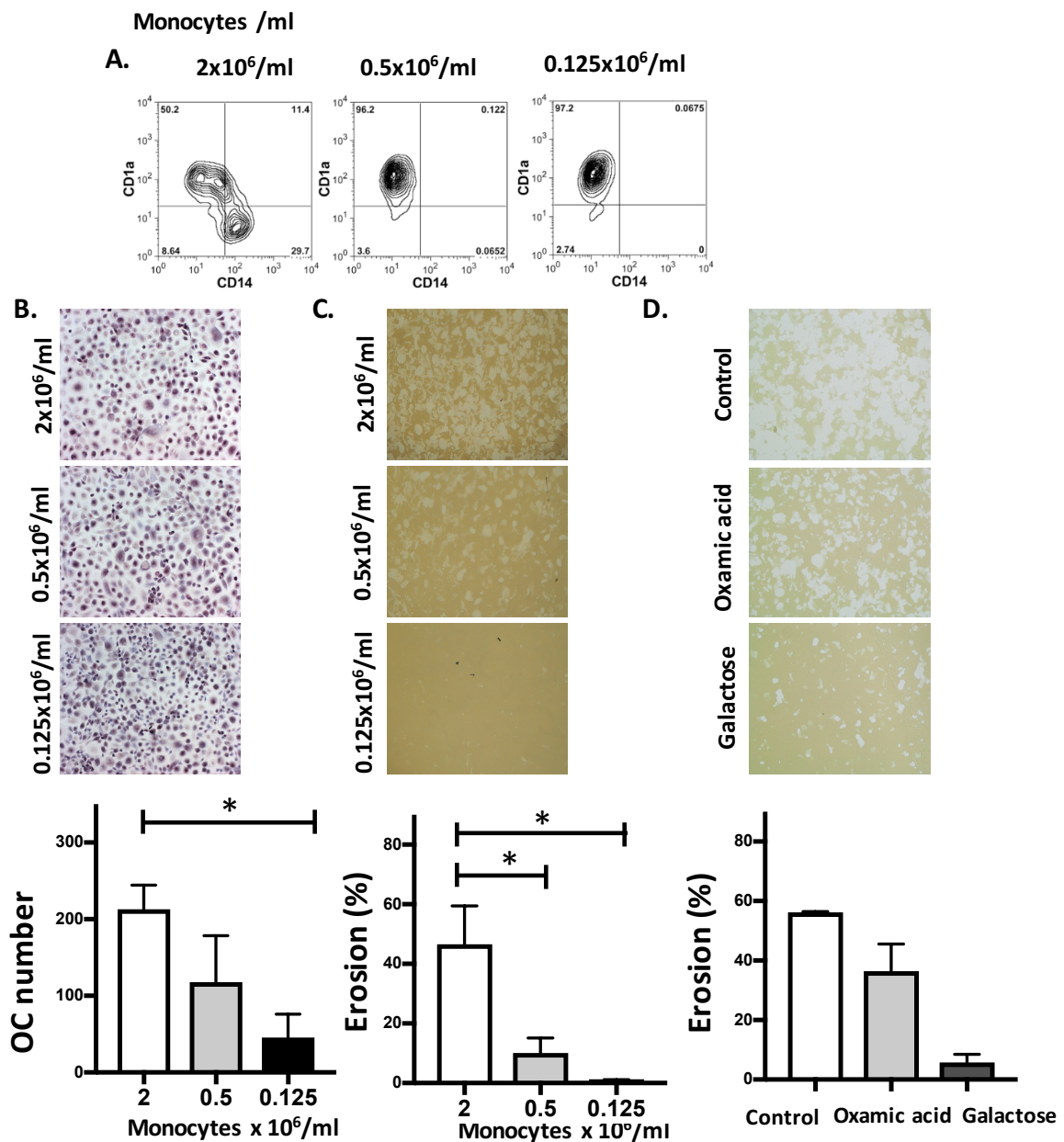
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Figure 15: Cell culture density influences iDC transdifferentiation in to OCs. (A) Contour plots represent CD14 and CD1a distribution on monocyte-derived DCs generated in dense or sparse cultures ($2x10^6$, $0.5x10^6$ and $0.125x10^6$ monocytes/ml). The different DC types were trandifferentiated into OCs in the presence of M-CSF and RANKL. (B) OC development was monitored with the help of TRAP staining (B) or a bone resorption assay using calcium-phosphate surface (C). OC development was also studied using DCs that developed in dense cultures in the presence of glycolysis inhibitors (D). *$p<0.05$.

In summary, we demonstrated that an increase in glycolytic activity that typically follows hypoxia, inflammation or tumor growth can downregulate the development of inflammatory DCs and increase DC-OC transdifferentiation. Previous studies have reported elevated lactate levels and decreased glucose concentrations in the SF of RA patients (229, 230), which might

be explained by the increased infiltration and activation of immune cells (231). Interestingly, an increased OC formation has been reported in hypoxia and the downregulation of HIF expression prevented experimental arthritis (231-234). Another important finding suggested a role for PAD enzymes in maintaining the functional plasticity iDCs to differentiate in to OCs and in the expression of citrullinated proteins. PAD enzyme expression is increased during hypoxia (235) and in inflammatory milieu (71). Our data indicated that lactic acid can reprogram DC development into an OC-prone pathway, which leads to increased PAD expression and protein citrullination, although the mechanistic link between glycolysis and protein citrullination remains to be understood (Paper III and IV). Protein citrullination might facilitate DC-OC transdifferentiation at steady state, and increase the targeting of the cells by ACPAs, which can potentially contribute to an accelerated bone erosion.

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5 CONCLUSION AND FUTURE PERSPECTIVES

In my thesis, I have focused on the molecular mechanisms underlying the effects of RA-associated autoantibodies on bone erosion. Previous studies have indicated that ACPA positive individuals can develop bone loss and pain in the absence of inflammation (152, 209). In our studies, we have established a link between adaptive immunity, i.e. autoantibodies, OC activation, bone destruction and pain.

We demonstrated that certain ACPA clones can promote osteoclastogenesis through an IL-8-mediated autocrine mechanism. ACPAs administered into mice could bind potential OC precursors in the bone marrow, induce local IL-8 production and pain triggered by IL-8 binding to CXCR2 receptors in the nociceptors. Treatment with reparixin, an IL-8 antagonist, modulated ACPA-induced bone loss and pain-like behaviour in mice. These findings provided novel insights to the roles of ACPAs in bone destruction and pain, suggesting a novel pathogenic scenario and therapeutic opportunities for the early stages of RA development in ACPA-positive individuals.

In the second part of the thesis (Paper I and IV), we have identified ACPA targets on the OC surface and we have found that protein citrullination by PAD enzymes is needed for both the steady state and the ACPA-mediated osteoclastogenesis. The expression of the citrullinated protein by OC precursors indicates that these cells can be targeted by ACPAs at the steady state, thus providing a potential explanation for ACPA-mediated bone loss in the absence of inflammation. Future studies are needed to address the exact contribution of PAD isozymes towards osteoclastogensis and the effects of PAD blocking in ACPA –dependent arthritis models. PAD inhibition or deficiency have been associated with decreased disease activity in collagen-induced or TNF-dependent arthritis models (81). PAD enzymes might therefore be important drug targets to prevent and treat both inflammation and ACPA-dependent bone loss in RA.

In the last part of my thesis (Paper III and IV), we have focused on the heterogeneity of DCs and their functional plasticity to develop into OCs. We have proposed that the increase of glycolytic metabolism and protein citrullination, both are characteristic of the inflamed joints during RA, can turn the developing DCs towards OC differentiation, thereby contributing to increased bone damage.
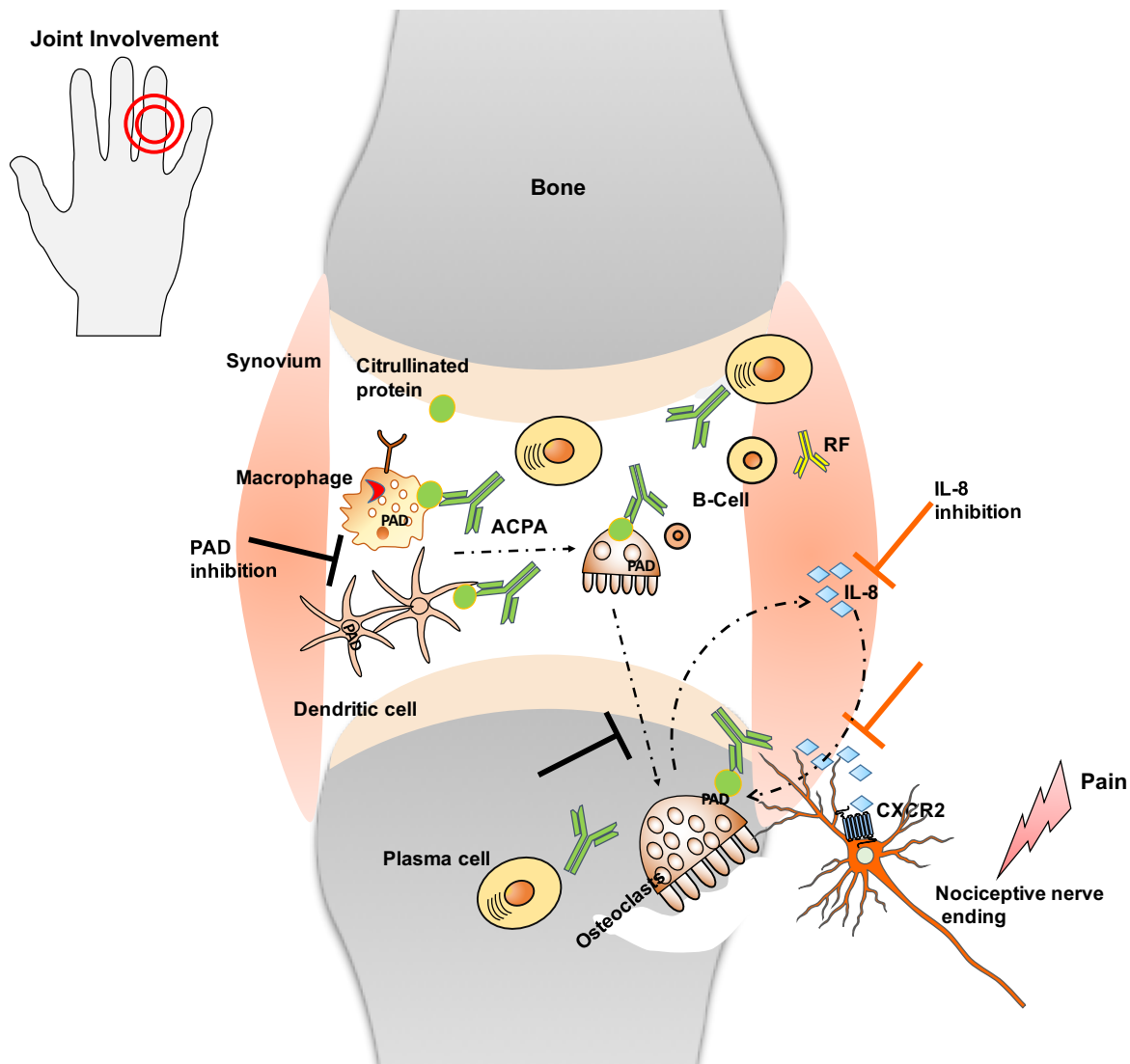
Figure 16: ACPA induced osteoclastogensis leads to bone destruction and pain-like behavior. ACPAs can bind MΦ and DC in the bone compartment and increase their differentiation into OC. IL-8 released during the differentiation process can activate the nociceptors and induce pain behaviour.

Our future research aims to understand the intracellular mechanisms induced by ACPAs in developing OCs and we need to identify the key cell surface targets triggered by these autoantibodies. We would also like to understand how RA develops in ACPA-positive individuals through modelling 'second hit' mechanisms, through introducing transient joint inflammation in ACPA-injected mice. We anticipate that ACPA-dependent murine arthritis models, resembling human ACPA positive RA, will increase our overall knowledge in disease progression and provide opportunity for pre-clinical drug trials. Our hope is that in the near future be able to prevent bone loss and pain in ACPA positive at-risk individuals by modulating IL-8 and the PAD enzymes

6 ACKNOWLEDGEMENT

7 REFERENCE

1. Scott D, Coulton B, Symmons D, Popert A. Long-term outcome of treating rheumatoid arthritis: results after 20 years. The Lancet. 1987;329(8542):1108-11.

2. Alarcón GS. Epidemiology of rheumatoid arthritis. Rheumatic diseases clinics of North America. 1995;21(3):589.

3. Kvien TK. Epidemiology and burden of illness of rheumatoid arthritis. Pharmacoeconomics. 2004;22(Supplement 1):1-12.

4. Techniques HL. East-West Anti-Aging Strategies. Joseph P Hou Ph D, Joseph P Hou AuthorHouse. 2010.

5. Lars K, Anca I, Stephen P. Rheumatoid arthritis: Seminar. Lancet. 2009;373:659-72.

6. Scott DL, Wolfe F. Huizinga TW. Rheumatoid arthritis Lancet. 2010;376:1094-108.

7. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis & Rheumatism. 1988;31(3):315-24.

8. Initiative C. 2010 rheumatoid arthritis classification criteria. Arthritis & Rheumatism. 2010;62(9):2569-81.

9. Malmström V, Catrina AI, Klareskog L. The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting. Nature Reviews Immunology. 2016.

10. Waaler E. On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. Apmis. 1940;17(2):172-88.

11. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid factors: clinical applications. Disease markers. 2013;35(6):727-34.

12. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis & Rheumatology. 1988;31(3):315-24.

13. Nienhuis R, Mandema E, Smids C. New serum factor in patients with rheumatoid arthritis: the antiperinuclear factor. Annals of the rheumatic diseases. 1964;23(4):302.

14. Simon M, Girbal E, Sebbag M, Gomes-Daudrix V, Vincent C, Salama G, et al. The cytokeratin filament-aggregating protein filaggrin is the target of the so-called" antikeratin antibodies," autoantibodies specific for rheumatoid arthritis. Journal of Clinical Investigation. 1993;92(3):1387.

15. Schellekens GA, de Jong BA, van den Hoogen FH, Van de Putte L, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. Journal of Clinical Investigation. 1998;101(1):273.

16. Schellekens GA, Visser H, De Jong BA, Van Den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis & Rheumatology. 2000;43(1):155-63.

17. Pruijn GJ, Wiik A, van Venrooij WJ. The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis. Arthritis research & therapy. 2010;12(1):203.

18. Lee Nelson J, Dugowson CE, Koepsell TD, Voigt LF, Branchaud AM, Barrington RA, et al. Rheumatoid factor, HLA–DR4, and allelic variants of DRB1 in women with recent-onset rheumatoid arthritis. Arthritis & Rheumatology. 1994;37(5):673-80.

19.		Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis & Rheumatology. 1987;30(11):1205-13.

20.		MacGregor A, Ollier W, Thomson W, Jawaheer D, Silman A. HLA-DRB1* 0401/0404 genotype and rheumatoid arthritis: increased association in men, young age at onset, and disease severity. The Journal of Rheumatology. 1995;22(6):1032-6.

21.		Huizinga TW, Amos CI, van der Helm-van Mil A, Chen W, Van Gaalen FA, Jawaheer D, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA–DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis & Rheumatology. 2005;52(11):3433-8.

22.		van der Helm-van Mil A, Verpoort KN, Breedveld FC, Huizinga TW, Toes RE, de Vries RR. The HLA–DRB1 shared epitope alleles are primarily a risk factor for anti–cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. Arthritis & Rheumatology. 2006;54(4):1117-21.

23.		Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. The American Journal of Human Genetics. 2004;75(2):330-7.

24.		Yarwood A, Huizinga TW, Worthington J. The genetics of rheumatoid arthritis: risk and protection in different stages of the evolution of RA. Rheumatology. 2014;55(2):199-209.

25.		Plenge RM, Padyukov L, Remmers EF, Purcell S, Lee AT, Karlson EW, et al. Replication of putative candidate-gene associations with rheumatoid arthritis in> 4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. The American Journal of Human Genetics. 2005;77(6):1044-60.

26.		Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nature genetics. 2003;34(4):395.

27.		Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. A gene–environment interaction between smoking and shared epitope genes in HLA–DR provides a high risk of seropositive rheumatoid arthritis. Arthritis & Rheumatism. 2004;50(10):3085-92.

28.		Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, et al. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. Annals of the rheumatic diseases. 2003;62(9):835-41.

29.		Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. A gene–environment interaction between smoking and shared epitope genes in HLA–DR provides a high risk of seropositive rheumatoid arthritis. Arthritis & Rheumatology. 2004;50(10):3085-92.

30.		Karlson EW, Chang S-C, Cui J, Chibnik LB, Fraser PA, DeVivo I, et al. Gene-environment interaction between HLA-DRB1 shared epitope and heavy cigarette smoking in predicting incident RA. Annals of the rheumatic diseases. 2009.

31.		Stolt P, Yahya A, Bengtsson C, Källberg H, Rönnelid J, Lundberg I, et al. Silica exposure among male current smokers is associated with a high risk of developing ACPA positive rheumatoid arthritis. Annals of the rheumatic diseases. 2009:ard. 2009.114694.

32.		Stolt P, Källberg H, Lundberg I, Sjögren B, Klareskog L, Alfredsson L. Silica exposure is associated with increased risk of developing rheumatoid arthritis: results from the Swedish EIRA study. Annals of the rheumatic diseases. 2005;64(4):582-6.

33.		Too CL, Muhamad NA, Ilar A, Padyukov L, Alfredsson L, Klareskog L, et al. Occupational exposure to textile dust increases the risk of rheumatoid arthritis: results from

a Malaysian population-based case–control study. Annals of the rheumatic diseases. 2016;75(6):997-1002.

34.   Hart JE, Källberg H, Laden F, Bellander T, Costenbader KH, Holmqvist M, et al. Ambient air pollution exposures and risk of rheumatoid arthritis: results from the Swedish EIRA case–control study. Annals of the rheumatic diseases. 2012:annrheumdis-2012-201587.

35.   Hart JE, Laden F, Puett RC, Costenbader KH, Karlson EW. Exposure to traffic pollution and increased risk of rheumatoid arthritis. Environmental health perspectives. 2009;117(7):1065.

36.   Catrina AI, Joshua V, Klareskog L, Malmström V. Mechanisms involved in triggering rheumatoid arthritis. Immunological reviews. 2016;269(1):162-74.

37.   Reynisdottir G, Karimi R, Joshua V, Olsen H, Hensvold AH, Harju A, et al. Structural changes and antibody enrichment in the lungs are early features of anti–citrullinated protein antibody–positive rheumatoid arthritis. Arthritis & rheumatology. 2014;66(1):31-9.

38.   Reynisdottir G, Olsen H, Joshua V, Engström M, Forsslund H, Karimi R, et al. Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis. Annals of the rheumatic diseases. 2015:annrheumdis-2015-208216.

39.   Willis VC, Demoruelle MK, Derber LA, Chartier-Logan CJ, Parish MC, Pedraza IF, et al. Sputum autoantibodies in patients with established rheumatoid arthritis and subjects at risk of future clinically apparent disease. Arthritis & Rheumatology. 2013;65(10):2545-54.

40.   Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Rönnelid J, et al. Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. Arthritis research & therapy. 2011;13(1):R13.

41.   Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis & Rheumatology. 2003;48(10):2741-9.

42.   Brink M, Verheul MK, Rönnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis research & therapy. 2015;17(1):25.

43.   van der Woude D, Rantapää-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Annals of the rheumatic diseases. 2010;69(8):1554-61.

44.   Bizzaro N, Bartoloni E, Morozzi G, Manganelli S, Riccieri V, Sabatini P, et al. Anti-cyclic citrullinated peptide antibody titer predicts time to rheumatoid arthritis onset in patients with undifferentiated arthritis: results from a 2-year prospective study. Arthritis research & therapy. 2013;15(1):R16.

45.   Brink M, Hansson M, Mathsson L, Jakobsson PJ, Holmdahl R, Hallmans G, et al. Multiplex analyses of antibodies against citrullinated peptides in individuals prior to development of rheumatoid arthritis. Arthritis Rheum. 2013;65(4):899-910.

46.   van der Woude D, Syversen SW, van der Voort EI, Verpoort KN, Goll GL, van der Linden MP, et al. The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis. Annals of the rheumatic diseases. 2010:annrheumdis116384.

47.   Johansson L, Pratesi F, Brink M, Ärlestig L, D'Amato C, Bartaloni D, et al. Antibodies directed against endogenous and exogenous citrullinated antigens pre-date the onset of rheumatoid arthritis. Arthritis research & therapy. 2016;18(1):127.

52

48.		Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunological reviews. 2010;233(1):233-55.

49.		Laurent L, Anquetil F, Clavel C, Ndongo-Thiam N, Offer G, Miossec P, et al. IgM rheumatoid factor amplifies the inflammatory response of macrophages induced by the rheumatoid arthritis-specific immune complexes containing anticitrullinated protein antibodies. Annals of the rheumatic diseases. 2015;74(7):1425-31.

50.		Manara M, Sinigaglia L. Bone and TNF in rheumatoid arthritis: clinical implications. RMD open. 2015;1(Suppl 1):e000065.

51.		Scott DL. Radiological progression in established rheumatoid arthritis. The Journal of Rheumatology Supplement. 2004;69:55-65.

52.		Müller-Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, et al. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. The American journal of pathology. 1996;149(5):1607.

53.		Deribe YL, Pawson T, Dikic I. Post-translational modifications in signal integration. Nature structural & molecular biology. 2010;17(6):666-72.

54.		Mann M, Jensen ON. Proteomic analysis of post-translational modifications. Nature biotechnology. 2003;21(3):255-61.

55.		Trouw LA, Rispens T, Toes RE. Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nature Reviews Rheumatology. 2017.

56.		Rogers G, Simmonds D. Content of citrulline and other amino-acids in a protein of hair follicles. Nature. 1958;182(4629):186-7.

57.		György B, Tóth E, Tarcsa E, Falus A, Buzás EI. Citrullination: a posttranslational modification in health and disease. The international journal of biochemistry & cell biology. 2006;38(10):1662-77.

58.		E Witalison E, R Thompson P, J Hofseth L. Protein arginine deiminases and associated citrullination: physiological functions and diseases associated with dysregulation. Current drug targets. 2015;16(7):700-10.

59.		Baka Z, György B, Géher P, Buzás EI, Falus A, Nagy G. Citrullination under physiological and pathological conditions. Joint Bone Spine. 2012;79(5):431-6.

60.		Hensvold AH, Reynisdottir G, Catrina AI. From citrullination to specific immunity and disease in rheumatoid arthritis. Protein Deimination in Human Health and Disease: Springer; 2014. p. 25-40.

61.		Christophorou MA, Castelo-Branco G, Halley-Stott RP, Oliveira CS, Loos R, Radzisheuskaya A, et al. Citrullination regulates pluripotency and histone H1 binding to chromatin. Nature. 2014.

62.		Mendez MG, Kojima S-I, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. The FASEB Journal. 2010;24(6):1838-51.

63.		Mastronardi FG, Wood DD, Mei J, Raijmakers R, Tseveleki V, Dosch H-M, et al. Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. Journal of Neuroscience. 2006;26(44):11387-96.

64.		Lazarus RC, Buonora JE, Flora MN, Freedy JG, Holstein GR, Martinelli GP, et al. Protein citrullination: a proposed mechanism for pathology in traumatic brain injury. Frontiers in neurology. 2015;6.

65.		Rogers GE, Harding HW, Llewellyn-Smith IJ. The origin of citrulline-containing proteins in the hair follicle and the chemical nature of trichohyalin, an intracellular precursor. Biochimica et Biophysica Acta (BBA)-Protein Structure. 1977;495(1):159-75.

66.   Kearney PL, Bhatia M, Jones NG, Yuan L, Glascock MC, Catchings KL, et al. Kinetic characterization of protein arginine deiminase 4: a transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. Biochemistry. 2005;44(31):10570-82.

67.   Pollmann S, Stensland M, Halvorsen EH, Sollid LM, Kvien TK, Fleckenstein B, et al. Anti-PAD4 autoantibodies in rheumatoid arthritis: levels in serum over time and impact on PAD4 activity as measured with a small synthetic substrate. Rheumatology international. 2012;32(5):1271-6.

68.   Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij D-J, et al. Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Annals of the rheumatic diseases. 2004;63(4):373-81.

69.   Damgaard D, Senolt L, Nielsen MF, Pruijn GJ, Nielsen CH. Demonstration of extracellular peptidylarginine deiminase (PAD) activity in synovial fluid of patients with rheumatoid arthritis using a novel assay for citrullination of fibrinogen. Arthritis research & therapy. 2014;16(6):498.

70.   Ferrari-Lacraz S, Sebbag M, Chicheportiche R, Foulquier C, Serre G, Dayer J-M. Contact with stimulated T cells up-regulates expression of peptidylarginine deiminase 2 and 4 by human monocytes. European cytokine network. 2012;23(2):36-44.

71.   Shelef MA, Sokolove J, Lahey LJ, Wagner CA, Sackmann EK, Warner TF, et al. Peptidylarginine deiminase 4 contributes to tumor necrosis factor α–induced inflammatory arthritis. Arthritis & rheumatology. 2014;66(6):1482-91.

72.   Mohanan S, Cherrington BD, Horibata S, McElwee JL, Thompson PR, Coonrod SA. Potential role of peptidylarginine deiminase enzymes and protein citrullination in cancer pathogenesis. Biochemistry research international. 2012;2012.

73.   Fan L, He D, Wang Q, Zong M, Zhang H, Yang L, et al. Citrullinated vimentin stimulates proliferation, pro-inflammatory cytokine secretion, and PADI4 and RANKL expression of fibroblast-like synoviocytes in rheumatoid arthritis. Scandinavian journal of rheumatology. 2012;41(5):354-8.

74.   Sun B, Dwivedi N, Bechtel TJ, Paulsen JL, Muth A, Bawadekar M, et al. Citrullination of NF-κB p65 promotes its nuclear localization and TLR-induced expression of IL-1β and TNFα. Science Immunology. 2017;2(12):eaal3062.

75.   Chumanevich AA, Causey CP, Knuckley BA, Jones JE, Poudyal D, Chumanevich AP, et al. Suppression of colitis in mice by Cl-amidine: a novel peptidylarginine deiminase inhibitor. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2011;300(6):G929-G38.

76.   Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. The Journal of cell biology. 2009;184(2):205-13.

77.   Knight JS, Zhao W, Luo W, Subramanian V, O'Dell AA, Yalavarthi S, et al. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. The Journal of clinical investigation. 2013;123(7):2981-93.

78.   Knight JS, Luo W, O'Dell AA, Yalavarthi S, Zhao W, Subramanian V, et al. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. Circulation research. 2014;114(6):947-56.

79.   Wang Y, Li P, Wang S, Hu J, Chen XA, Wu J, et al. Anticancer peptidylarginine deiminase (PAD) inhibitors regulate the autophagy flux and the mammalian target of rapamycin complex 1 activity. Journal of Biological Chemistry. 2012;287(31):25941-53.

80.	Moscarello MA, Lei H, Mastronardi FG, Winer S, Tsui H, Li Z, et al. Inhibition of peptidyl-arginine deiminases reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. Disease models & mechanisms. 2013;6(2):467-78.

81.	Willis VC, Gizinski AM, Banda NK, Causey CP, Knuckley B, Cordova KN, et al. N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. The Journal of Immunology. 2011;186(7):4396-404.

82.	Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. Journal of internal medicine. 2007;261(6):511-28.

83.	Catrina AI, Ytterberg AJ, Reynisdottir G, Malmström V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nature reviews Rheumatology. 2014;10(11):645-53.

84.	Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. Journal of autoimmunity. 2010;34(3):J258-J65.

85.	Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, et al. Effects of cigarette smoke on the human airway epithelial cell transcriptome. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(27):10143-8.

86.	Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. CHEST Journal. 1999;115(3):829-35.

87.	Sebastian A, Pehrson C, Larsson L. Elevated concentrations of endotoxin in indoor air due to cigarette smoking. Journal of environmental monitoring. 2006;8(5):519-22.

88.	Robbins CS, Franco F, Mouded M, Cernadas M, Shapiro SD. Cigarette smoke exposure impairs dendritic cell maturation and T cell proliferation in thoracic lymph nodes of mice. The Journal of Immunology. 2008;180(10):6623-8.

89.	Makrygiannakis D, Hermansson M, Ulfgren A-K, Nicholas AP, Zendman AJ, Eklund A, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Annals of the rheumatic diseases. 2008;67(10):1488-92.

90.	Ireland JM, Unanue ER. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. The Journal of experimental medicine. 2011;208(13):2625-32.

91.	Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. science. 2004;303(5663):1532-5.

92.	Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. Journal of Experimental Medicine. 2010:jem. 20100239.

93.	Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Science translational medicine. 2016;8(369):369ra176-369ra176.

94.	Pap T, Müller-Ladner U, Gay RE, Gay S. Fibroblast biology: role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. Arthritis Research & Therapy. 2000;2(5):361.

95.	Siebert S, Tsoukas A, Robertson J, McInnes I. Cytokines as therapeutic targets in rheumatoid arthritis and other inflammatory diseases. Pharmacological reviews. 2015;67(2):280-309.

96.	Scally SW, Petersen J, Law SC, Dudek NL, Nel HJ, Loh KL, et al. A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis. Journal of Experimental Medicine. 2013;210(12):2569-82.

97.	Gerstner C, Dubnovitsky A, Sandin C, Kozhukh G, Uchtenhagen H, James EA, et al. Functional and structural characterization of a novel hla-DrB1* 04: 01-restricted α-enolase T cell epitope in rheumatoid arthritis. Frontiers in immunology. 2016;7.

98.	Lundberg K, Bengtsson C, Kharlamova N, Reed E, Jiang X, Kallberg H, et al. Genetic and environmental determinants for disease risk in subsets of rheumatoid arthritis defined by the anticitrullinated protein/peptide antibody fine specificity profile. Annals of the rheumatic diseases. 2012:annrheumdis-2012-201484.

99.	Tan YC, Kongpachith S, Blum LK, Ju CH, Lahey LJ, Lu DR, et al. Barcode-Enabled Sequencing of Plasmablast Antibody Repertoires in Rheumatoid Arthritis. Arthritis & rheumatology. 2014;66(10):2706-15.

100.	Corsiero E, Bombardieri M, Carlotti E, Pratesi F, Robinson W, Migliorini P, et al. Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs. Annals of the rheumatic diseases. 2015:annrheumdis-2015-208356.

101.	Amara K, Steen J, Murray F, Morbach H, Fernandez-Rodriguez BM, Joshua V, et al. Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition. The Journal of experimental medicine. 2013;210(3):445-55.

102.	Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. The Journal of clinical investigation. 2012;122(5):1791-802.

103.	Harre U, Lang SC, Pfeifle R, Rombouts Y, Frühbeißer S, Amara K, et al. Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss. Nature communications. 2015;6.

104.	Pfeifle R, Rothe T, Ipseiz N, Scherer HU, Culemann S, Harre U, et al. Regulation of autoantibody activity by the IL-23-TH17 axis determines the onset of autoimmune disease. Nature immunology. 2017;18(1):104-13.

105.	Sokolove J, Johnson DS, Lahey LJ, Wagner CA, Cheng D, Thiele GM, et al. Rheumatoid factor as a potentiator of anti-citrullinated protein antibody-mediated inflammation in rheumatoid arthritis. Arthritis Rheumatol. 2014;66(4):813-21.

106.	Zhao X, Okeke NL, Sharpe O, Batliwalla FM, Lee AT, Ho PP, et al. Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. Arthritis Research and Therapy. 2008;10(4):R94.

107.	Gallagher RM. Biopsychosocial pain medicine and mind-brain-body science. Physical medicine and rehabilitation clinics of North America. 2004;15(4):855-82.

108.	Schaible H-G, Richter F, Ebersberger A, Boettger MK, Vanegas H, Natura G, et al. Joint pain. Experimental brain research. 2009;196(1):153-62.

109.	McDougall JJ. Arthritis and pain. Neurogenic origin of joint pain. Arthritis research & therapy. 2006;8(6):220.

110.	Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nature reviews Neuroscience. 2005;6(7):521.

111.	Van Hecke O, Austin SK, Khan RA, Smith B, Torrance N. Neuropathic pain in the general population: a systematic review of epidemiological studies. PAIN®. 2014;155(4):654-62.

112.	Derksen V, Huizinga T, van der Woude D, editors. The role of autoantibodies in the pathophysiology of rheumatoid arthritis. Seminars in Immunopathology; 2017: Springer.

113.	van der Helm-van Mil A, Landewé R. Appropriate use of the EULAR definition of arthralgia suspicious for progression to rheumatoid arthritis. Annals of the rheumatic diseases. 2017;76(6):e15-e.

114.	Burgers LE, van Steenbergen HW, ten Brinck RM, Huizinga TW, van der Helm-van AH. Differences in the symptomatic phase preceding ACPA-positive and ACPA-

negative RA: a longitudinal study in arthralgia during progression to clinical arthritis. Annals of the Rheumatic Diseases. 2017:annrheumdis-2017-211325.

115.   Altawil R, Saevarsdottir S, Wedrén S, Alfredsson L, Klareskog L, Lampa J. Remaining pain in early rheumatoid arthritis patients treated with methotrexate. Arthritis care & research. 2016;68(8):1061-8.

116.   Borenstein D, Altman R, Bello A, Chatham W, Clauw D, Crofford L, et al. Report of the American college of rheumatology pain management task force. Arthritis Care and Research. 2010;62(5):590-9.

117.   Schaible H-G. Nociceptive neurons detect cytokines in arthritis. Arthritis research & therapy. 2014;16(5):470.

118.   Qu L, Zhang P, LaMotte RH, Ma C. Neuronal Fc-gamma receptor I mediated excitatory effects of IgG immune complex on rat dorsal root ganglion neurons. Brain, behavior, and immunity. 2011;25(7):1399-407.

119.   Rech J, Hess A, Finzel S, Kreitz S, Sergeeva M, Englbrecht M, et al. Association of brain functional magnetic resonance activity with response to tumor necrosis factor inhibition in rheumatoid arthritis. Arthritis & Rheumatology. 2013;65(2):325-33.

120.   Roche PA, Klestov AC, Heim HM. Description of stable pain in rheumatoid arthritis: a 6 year study. The Journal of rheumatology. 2003;30(8):1733-8.

121.   Orr C, Sousa E, Boyle DL, Buch MH, Buckley CD, Cañete JD, et al. Synovial tissue research: a state-of-the-art review. Nature reviews Rheumatology. 2017;13(8):463-75.

122.   Smith M, Barg E, Weedon H, Papengelis V, Smeets T, Tak P, et al. Microarchitecture and protective mechanisms in synovial tissue from clinically and arthroscopically normal knee joints. Annals of the rheumatic diseases. 2003;62(4):303-7.

123.   Burmester GR, Locher P, Koch B, Winchester RJ, Dimitriu-Bona A, Kalden JR, et al. The tissue architecture of synovial membranes in inflammatory and non-inflammatory joint diseases. Rheumatology International. 1983;3(4):173-81.

124.   Athanasou NA, Quinn J, Heryet A, Puddle B, Woods CG, McGee JOD. The immunohistology of synovial lining cells in normal and inflamed synovium. The Journal of pathology. 1988;155(2):133-42.

125.   Hitchon CA, El-Gabalawy HS. Suppl 1: The Synovium in Rheumatoid Arthritis. The open rheumatology journal. 2011;5:107.

126.   Gerlag DM, Tak PP. How to perform and analyse synovial biopsies. Best Practice & Research Clinical Rheumatology. 2009;23(2):221-32.

127.   Hogg N, Palmer DG, Revell PA. Mononuclear phagocytes of normal and rheumatoid synovial membrane identified by monoclonal antibodies. Immunology. 1985;56(4):673.

128.   Cravens PD, Lipsky PE. Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. Immunology and cell biology. 2002;80(5):497-505.

129.   Highton J, Carlisle B, Palmer DG. Changes in the phenotype of monocytes/macrophages and expression of cytokine mRNA in peripheral blood and synovial fluid of patients with rheumatoid arthritis. Clinical & Experimental Immunology. 1995;102(3):541-6.

130.   Cope A. Studies of T-cell activation in chronic inflammation. Arthritis Res. 2002;4(Suppl 3):S197-S211.

131.   Dörner T, Burmester GR. The role of B cells in rheumatoid arthritis: mechanisms and therapeutic targets. Current opinion in rheumatology. 2003;15(3):246.

132.   Ng C, Biniecka M, Kennedy A, McCormick J, Fitzgerald O, Bresnihan B, et al. Synovial tissue hypoxia and inflammation in vivo. Annals of the rheumatic diseases. 2010:annrheumdis119776.

133.   Tak PP, Taylor PC, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, et al. Decrease in cellularity and expression of adhesion molecules by anti–tumor necrosis factor

α monoclonal antibody treatment in patients with rheumatoid arthritis. Arthritis & Rheumatology. 1996;39(7):1077-81.

134.	Revu S, Neregård P, af Klint E, Korotkova M, Catrina AI. Synovial membrane immunohistology in early-untreated rheumatoid arthritis reveals high expression of catabolic bone markers that is modulated by methotrexate. Arthritis research & therapy. 2013;15(6):R205.

135.	Decker B, McKenzie BF, McGuckin WF, Slocumb CH. Comparative distribution of proteins and glycoproteins of serum and synovial fluid. Arthritis & Rheumatology. 1959;2(2):162-77.

136.	McInnes IB, Buckley CD, Isaacs JD. Cytokines in rheumatoid arthritis--shaping the immunological landscape. Nature Reviews Rheumatology. 2016;12(1):63.

137.	Brennan FM, Jackson A, Chantry D, Maini R, Feldmann M. Inhibitory effect of TNF [alpha] antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. The Lancet. 1989;334(8657):244-7.

138.	Epstein FH. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med. 2001;344(12).

139.	Miossec P. An update on the cytokine network in rheumatoid arthritis. Current opinion in rheumatology. 2004;16(3):218.

140.	Arend WP. Physiology of cytokine pathways in rheumatoid arthritis. Arthritis Care & Research. 2001;45(1):101-6.

141.	Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. The Journal of experimental medicine. 2006;203(12):2673.

142.	Hamilton Oa, Filonzi El, Ianches G. Regulation of macrophage colony-stimulating factor (M-CSF) production in cultured human synovial fibroblasts. Growth Factors. 1993;9(2):157-65.

143.	Udalova I, Mantovani A, Feldmann M. Macrophage heterogeneity in the context of rheumatic diseases. Nature Reviews Rheumatology. 2016;12:472-85.

144.	Smeets TJ, Kraan MC, van Loon ME, Tak PP. Tumor necrosis factor α blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue. Arthritis & Rheumatology. 2003;48(8):2155-62.

145.	Vigna Pérez M, Abud Mendoza C, Portillo Salazar H, Alvarado Sánchez B, Cuevas Orta E, Moreno Valdés R, et al. Immune effects of therapy with Adalimumab in patients with rheumatoid arthritis. Clinical & Experimental Immunology. 2005;141(2):372-80.

146.	Zwerina J, Redlich K, Schett G, Smolen JS. Pathogenesis of rheumatoid arthritis: targeting cytokines. Annals of the New York Academy of Sciences. 2005;1051(1):716-29.

147.	Deal C. Bone loss in rheumatoid arthritis: systemic, periarticular, and focal. Current rheumatology reports. 2012;14(3):231-7.

148.	Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis & Rheumatology. 2000;43(2):250-8.

149.	Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-α. Endocrinology. 2000;141(11):3956-64.

150.	Horowitz M, Vignery A, Gershon RK, Baron R. Thymus-derived lymphocytes and their interactions with macrophages are required for the production of osteoclast-activating factor in the mouse. Proceedings of the National Academy of Sciences. 1984;81(7):2181-5.

58

151.   Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis & Rheumatology. 2000;43(2):259-69.

152.   Kleyer A, Finzel S, Rech J, Manger B, Krieter M, Faustini F, et al. Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. Annals of the rheumatic diseases. 2013:annrheumdis-2012-202958.

153.   Lems W, Dijkmans B. Should we look for osteoporosis in patients with rheumatoid arthritis? Annals of the rheumatic diseases. 1998;57(6):325-7.

154.   Hafez EA, Mansour HE, Hamza SH, Moftah SG, Younes TB, Ismail MA. Bone mineral density changes in patients with recent-onset rheumatoid arthritis. Clinical medicine insights Arthritis and musculoskeletal disorders. 2011;4:87.

155.   Van Staa T, Geusens P, Bijlsma J, Leufkens H, Cooper C. Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis. Arthritis & Rheumatology. 2006;54(10):3104-12.

156.   Güler-Yüksel M, Bijsterbosch J, Goekoop-Ruiterman Y, Breedveld F, Allaart C, de Vries-Bouwstra J, et al. Bone mineral density in patients with recently diagnosed, active rheumatoid arthritis. Annals of the rheumatic diseases. 2007;66(11):1508-12.

157.   van Schaardenburg D, Nielen MM, Lems WF, Twisk JW, Reesink HW, van de Stadt RJ, et al. Bone metabolism is altered in preclinical rheumatoid arthritis. Annals of the rheumatic diseases. 2011;70(6):1173-4.

158.   Steeve K, Marc P, Sandrine T, Dominique H, Yannick F. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine & Growth Factor Reviews. 2004;15(1):49-60.

159.   McArdle A, Marecic O, Tevlin R, Walmsley GG, Chan CK, Longaker MT, et al. The role and regulation of osteoclasts in normal bone homeostasis and in response to injury. Plastic and reconstructive surgery. 2015;135(3):808-16.

160.   Gravallese EM, Harada Y, Wang J-T, Gorn AH, Thornhill TS, Goldring SR. Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. The American journal of pathology. 1998;152(4):943.

161.   Halleen JM, Ylipahkala H, Alatalo SL, Janckila AJ, Heikkinen JE, Suominen H, et al. Serum tartrate-resistant acid phosphatase 5b, but not 5a, correlates with other markers of bone turnover and bone mineral density. Calcified tissue international. 2002;71(1):20.

162.   Kirstein B, Chambers TJ, Fuller K. Secretion of tartrate resistant acid phosphatase by osteoclasts correlates with resorptive behavior. Journal of cellular biochemistry. 2006;98(5):1085-94.

163.   Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, et al. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. Journal of Biological Chemistry. 1996;271(21):12511-6.

164.   Reinholt FP, Widholm SM, Ek Rylander B, Andersson G. Ultrastructural localization of a tartrate resistant acid ATPase in bone. Journal of bone and mineral research. 1990;5(10):1055-61.

165.   Halleen JM, Räisänen S, Salo JJ, Reddy SV, Roodman GD, Hentunen TA, et al. Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. Journal of Biological Chemistry. 1999;274(33):22907.

166.   Takahashi N, Udagawa N, Suda T. Vitamin D endocrine system and osteoclasts. BoneKEy reports. 2014;3.

167.   TAKAHASHI N, YAMANA H, YOSHIKI S, ROODMAN GD, MUNDY GR, JONES SJ, et al. Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology. 1988;122(4):1373-82.

168.	Raisz L, Trummel C, Holick M, DeLuca Hl. 1, 25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science. 1972;175(4023):768-9.

169.	Swarthout JT, D'Alonzo RC, Selvamurugan N, Partridge NC. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. Gene. 2002;282(1):1-17.

170.	Aslan D, Andersen MD, Gede LB, de Franca TK, Jørgensen SR, Schwarz P, et al. Mechanisms for the bone anabolic effect of parathyroid hormone treatment in humans. Scandinavian journal of clinical and laboratory investigation. 2012;72(1):14-22.

171.	Davey RA, Findlay DM. Calcitonin: physiology or fantasy? Journal of Bone and Mineral Research. 2013;28(5):973-9.

172.	Sato K, Takayanagi H. Osteoclasts, rheumatoid arthritis, and osteoimmunology. Current opinion in rheumatology. 2006;18(4):419.

173.	Takayanagi H. Inflammatory bone destruction and osteoimmunology. Journal of periodontal research. 2005;40(4):287-93.

174.	Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, et al. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the National Academy of Sciences. 1990;87(18):7260.

175.	Rivollier A, Mazzorana M, Tebib J, Piperno M, Aitsiselmi T, Rabourdin-Combe C, et al. Immature dendritic cell transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment. Blood. 2004;104(13):4029-37.

176.	Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest. 2005;115(2):282-90.

177.	Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, Stanley ER, et al. Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. Journal of Clinical Investigation. 1993;91(1):257.

178.	Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. Journal of Experimental Medicine. 1999;190(12):1741-54.

179.	Hodge JM, Kirkland MA, Nicholson GC. Multiple roles of M-CSF in human osteoclastogenesis. Journal of cellular biochemistry. 2007;102(3):759-68.

180.	Kim JH, Kim N. Signaling pathways in osteoclast differentiation. Chonnam medical journal. 2016;52(1):12-7.

181.	D'Amico L, Roato I. Cross-talk between T cells and osteoclasts in bone resorption. BoneKEy reports. 2012;1(6).

182.	Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S-i, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proceedings of the National Academy of Sciences. 1998;95(7):3597-602.

183.	Usui M, Xing L, Drissi H, Zuscik M, O'Keefe R, Chen D, et al. Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. Journal of Bone and Mineral Research. 2008;23(3):314-25.

184.	Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nature medicine. 2011;17(10):1231-4.

185.	Kim K-W, Cho M-L, Oh H-J, Kim H-R, Kang C-M, Heo Y-M, et al. TLR-3 enhances osteoclastogenesis through upregulation of RANKL expression from fibroblast-like synoviocytes in patients with rheumatoid arthritis. Immunology letters. 2009;124(1):9-17.

186.   Nakao A, Fukushima H, Kajiya H, Ozeki S, Okabe K. RANKL-stimulated TNF [alpha] production in osteoclast precursor cells promotes osteoclastogenesis by modulating RANK signaling pathways. Biochemical and biophysical research communications. 2007;357(4):945-50.

187.   Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, et al. Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology. 2000;141(9):3478-84.

188.   Walsh N, Gravallese E. Bone remodeling in rheumatic disease: a question of balance. Immunological reviews. 2010;233(1):301-12.

189.   Schett G, Gravallese E. Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. Nature Reviews Rheumatology. 2012;8(11):656-64.

190.   Goldring SR. Inflammatory signaling induced bone loss. Bone. 2015;80:143-9.

191.   Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ. Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. Bone. 2003;33(1):28-37.

192.   Kopesky P, Tiedemann K, Alkekhia D, Zechner C, Millard B, Schoeberl B, et al. Autocrine signaling is a key regulatory element during osteoclastogenesis. Biology open. 2014;3(8):767-76.

193.   Rothe L, Collin-Osdoby P, Chen Y, Sunyer T, Chaudhary L, Tsay A, et al. Human osteoclasts and osteoclast-like cells synthesize and release high basal and inflammatory stimulated levels of the potent chemokine interleukin-8. Endocrinology. 1998;139(10):4353-63.

194.   Kamalakar A, Bendre MS, Washam CL, Fowler TW, Carver A, Dilley JD, et al. Circulating interleukin-8 levels explain breast cancer osteolysis in mice and humans. Bone. 2014;61:176-85.

195.   Humphrey MB, Nakamura MC. A comprehensive review of immunoreceptor regulation of osteoclasts. Clinical reviews in allergy & immunology. 2016;51(1):48-58.

196.   Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nature reviews Immunology. 2007;7(4):292.

197.   Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, et al. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. Arthritis & Rheumatology. 2001;44(5):1003-12.

198.   Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. Journal of Experimental Medicine. 2006;203(12):2673-82.

199.   Buchwald ZS, Kiesel JR, DiPaolo R, Pagadala MS, Aurora R. Osteoclast activated FoxP3+ CD8+ T-cells suppress bone resorption in vitro. PloS one. 2012;7(6):e38199.

200.   Wu Y, Humphrey MB, Nakamura MC. Osteoclasts—the innate immune cells of the bone. Autoimmunity. 2008;41(3):183-94.

201.   Choi Y, Mi Woo K, Ko SH, Jung Lee Y, Park SJ, Kim HM, et al. Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8+ T cells. European journal of immunology. 2001;31(7):2179-88.

202.   Yeo L, Lom H, Juarez M, Snow M, Buckley C, Filer A, et al. Expression of FcRL4 defines a pro-inflammatory, RANKL-producing B cell subset in rheumatoid arthritis. Annals of the rheumatic diseases. 2015;74(5):928-35.

203.   Kocijan R, Harre U, Schett G. ACPA and Bone Loss in Rheumatoid Arthritis. Current rheumatology reports. 2013;15(10):1-5.

204.   Mok CC. Rituximab for the treatment of rheumatoid arthritis: an update. Drug design, Development and therapy. 2014;8:87.

205.	Boumans MJ, Thurlings RM, Yeo L, Scheel-Toellner D, Vos K, Gerlag DM, et al. Rituximab abrogates joint destruction in rheumatoid arthritis by inhibiting osteoclastogenesis. Annals of the rheumatic diseases. 2011:annrheumdis-2011-200198.

206.	Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. New England Journal of Medicine. 2003;349(20):1907-15.

207.	Axmann R, Herman S, Zaiss M, Franz S, Polzer K, Zwerina J, et al. CTLA-4 directly inhibits osteoclast formation. Annals of the rheumatic diseases. 2008;67(11):1603-9.

208.	Ossipova E, Cerqueira CF, Reed E, Kharlamova N, Israelsson L, Holmdahl R, et al. Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint. Arthritis research & therapy. 2014;16(4):R167.

209.	van de Stadt LA, van der Horst AR, de Koning MH, Bos WH, Wolbink GJ, van de Stadt RJ, et al. The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. Annals of the rheumatic diseases. 2010:annrheumdis132662.

210.	Nam JL, Hunt L, Hensor EM, Emery P. Enriching case selection for imminent RA: the use of anti-CCP antibodies in individuals with new non-specific musculoskeletal symptoms–a cohort study. Annals of the rheumatic diseases. 2015:annrheumdis-2015-207871.

211.	Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis research & therapy. 2005;8(1):1.

212.	Gallois A, Lachuer J, Yvert G, Wierinckx A, Brunet F, Rabourdin-Combe C, et al. Genome-wide expression analyses establish dendritic cells as a new osteoclast precursor able to generate bone-resorbing cells more efficiently than monocytes. Journal of Bone and Mineral Research. 2010;25(3):661-72.

213.	Coutant F, Miossec P. Altered dendritic cell functions in autoimmune diseases: distinct and overlapping profiles. Nature Reviews Rheumatology. 2016;12(12):703-15.

214.	Hansson M, Mathsson L, Schlederer T, Israelsson L, Matsson P, Nogueira L, et al. Validation of a multiplex chip-based assay for the detection of autoantibodies against citrullinated peptides. Arthritis research & therapy. 2012;14(5):R201.

215.	Makrygiannakis D, Revu S, Engström M, Af Klint E, Nicholas AP, Pruijn GJ, et al. Local administration of glucocorticoids decreases synovial citrullination in rheumatoid arthritis. Arthritis research & therapy. 2012;14(1):R20.

216.	Makrygiannakis D, af Klint E, Lundberg IE, Lofberg R, Ulfgren AK, Klareskog L, et al. Citrullination is an inflammation-dependent process. Ann Rheum Dis. 2006;65(9):1219-22.

217.	Zhang Z-J, Cao D-L, Zhang X, Ji R-R, Gao Y-J. Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. PAIN®. 2013;154(10):2185-97.

218.	Sokolove J, Zhao X, Chandra PE, Robinson WH. Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcγ receptor. Arthritis & Rheumatism. 2011;63(1):53-62.

219.	Negishi-Koga T, Gober H-J, Sumiya E, Komatsu N, Okamoto K, Sawa S, et al. Immune complexes regulate bone metabolism through FcRγ signalling. Nature communications. 2015;6.

220.	Hecht C, Englbrecht M, Rech J, Schmidt S, Araujo E, Engelke K, et al. Additive effect of anti-citrullinated protein antibodies and rheumatoid factor on bone erosions in patients with RA. Annals of the rheumatic diseases. 2014:annrheumdis-2014-205428.

221.   Van Steenbergen H, Ajeganova S, Forslind K, Svensson B, Van Der Helm-van Mil A. The effects of rheumatoid factor and anticitrullinated peptide antibodies on bone erosions in rheumatoid arthritis. Annals of the rheumatic diseases. 2015;74(1):e3-e.

222.   van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti–citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis & Rheumatology. 2011;63(11):3226-33.

223.   Brink M, Hansson M, Mathsson L, Jakobsson PJ, Holmdahl R, Hallmans G, et al. Multiplex analyses of antibodies against citrullinated peptides in individuals prior to development of rheumatoid arthritis. Arthritis & Rheumatism. 2013;65(4):899-910.

224.   Paleolog EM. The vasculature in rheumatoid arthritis: cause or consequence? International journal of experimental pathology. 2009;90(3):249-61.

225.   Treuhaft PS, McCarty DJ. Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. Arthritis & Rheumatology. 1971;14(4):475-84.

226.   Geborek P, Saxne T, Pettersson H, Wollheim F. Synovial fluid acidosis correlates with radiological joint destruction in rheumatoid arthritis knee joints. The Journal of rheumatology. 1989;16(4):468-72.

227.   Page G, Lebecque S, Miossec P. Anatomic localization of immature and mature dendritic cells in an ectopic lymphoid organ: correlation with selective chemokine expression in rheumatoid synovium. The Journal of Immunology. 2002;168(10):5333-41.

228.   Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. Blood. 2006;107(5):2013-21.

229.   Ciurtin C, Cojocaru VM, Miron IM, Preda F, Milicescu M, Bojincă M, et al. Correlation between different components of synovial fluid and pathogenesis of rheumatic diseases. Romanian journal of internal medicine= Revue roumaine de medecine interne. 2006;44(2):171-81.

230.   Gobelet C, Gerster J. Synovial fluid lactate levels in septic and non-septic arthritides. Annals of the Rheumatic diseases. 1984;43(5):742-5.

231.   Gaber T, Dziurla R, Tripmacher R, Burmester GR, Buttgereit F. Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! Annals of the rheumatic diseases. 2005;64(7):971-80.

232.   Knowles H, Athanasou N. Hypoxia-inducible factor is expressed in giant cell tumour of bone and mediates paracrine effects of hypoxia on monocyte–osteoclast differentiation via induction of VEGF. The Journal of pathology. 2008;215(1):56-66.

233.   Utting JC, Flanagan AM, Brandao-Burch A, Orriss IR, Arnett TR. Hypoxia stimulates osteoclast formation from human peripheral blood. Cell biochemistry and function. 2010;28(5):374-80.

234.   Ryu J-H, Chae C-S, Kwak J-S, Oh H, Shin Y, Huh YH, et al. Hypoxia-inducible factor-2α is an essential catabolic regulator of inflammatory rheumatoid arthritis. PLoS biology. 2014;12(6):e1001881.

235.   Sase T, Arito M, Onodera H, Omoteyama K, Kurokawa MS, Kagami Y, et al. Hypoxia-induced production of peptidylarginine deiminases and citrullinated proteins in malignant glioma cells. Biochemical and biophysical research communications. 2017;482(1):50-6.