M. Sci. Thesis in molecular biology

Human articular chondrocytes express Chemerin receptor, ChemR23, which conveys inflammatory signalling

---

Vivian Berg

December 2008

Department of Medical Biochemistry

Institute of Medical Biology

University of Tromsø

Norway

Acknowledgements

The present work was carried out at the Department of Medical Biochemistry, University Hospital of North-Norway, while I was a student at the Department of Medical Biochemistry, Institute of Medical Biology, University of Tromsø, Norway.

I would like to thank my supervisors, Yngve Figenschau, Jan Brox and Baldur Sveinbjørnsson for their guidance and support throughout this study. I would especially like to thank my Department and the head of department Majbrit Kragh for encouraging me and giving me the opportunity to carry out this study.

Thank you.

Table of content

Summary

Common features of arthritis include destruction of extracellular matrices in cartilage and bone as a result of chronic inflammation. Cartilage deterioration is generally described as a result from the effect of immune cells and their inflammatory mediators. However, recent reports suggest a role of chondrocytes in the initiation of inflammation in joints, and that they play a pivotal role in the destruction of their own matrix. Chondrocytes express multiple immune receptors and can produce and bind several cytokines, thus rendering them possible targets for therapy. We have demonstrated that serially cultured human articular chondrocytes posses the Chemerin receptor, ChemR23, and that this receptor is also present on chondrocytes in native human cartilage. In cultured chondrocytes we detected mRNA for the Chemerin receptor, and observed that stimulation with Chemerin resulted in phosphorylation of p44/p42 MAPK and Akt. Moreover, the Chemerin stimulation resulted in a marked increase of the pro-inflammatory cytokines IL-6 and IL-8, a modest increase in TNF-α and IL-1β, and marked increase in MMP-2, MMP-3 and MMP-13 in the culture supernatants. These results show that ChemR23 conveys pro-inflammatory signalling and affects MMP production when binding its ligand Chemerin. These observations indicate that ChemR23 takes part in inflammation initiation and cartilage degradation in joint disease.

Abbreviations

AA – Aracidonic acid
ACT – Autologous chondrocyte transplantation
APC – Antigen presenting cell
BLT1 – Leukotriene B4 receptor
cAMP – Cyclic adenosine monophosphate
CMKLR1 – Chemokine-Like Receptor 1
COX-2 – Cyclooxygenase-2
DC – Dendritic cell
DHA – Docosahexaenoic acid
EPA – Eicosapentaenoic acid
GAG – Glycosaminoglycan
GDP – Guanosine diphosphate
GPCR – G-protein coupled receptor
GTP – Guanosine triphosphate
IL-1β – Interleukine 1 beta
IL-8 – Interleukine 8
IL-6 – Interleukine 6
LO – Lipoxygenase
LTB4 – Leukotriene B4
MHC-II – Major Histoconpatibillity complex II
MMP – Matrix metalloproteinases
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell – Natural killer cell
OA – Osteoarthritis
PMN – Polymorphonuclear leukocyte
RA – Rheumatoid Arthritis
TIMP – Tissue inhibitor of metalloprotinases
TLR – Toll-Like Receptor
TNF-α – Tissue Necrosis Factor alpha

1. Introduction

Joint disease arises and develops mainly from arthritis, also termed joint inflammation. Arthritis comprises a group of conditions leading to damage of the joints and these conditions are often called articular degenerative inflammatory diseases [1]. Arthritic conditions include the diseases osteoarthritis, rheumatoid arthritis, psoriasis arthritis, septic arthritis and gout, however osteoarthritis (OA) and rheumatoid arthritis (RA) are the two most common types of arthritis world wide [2]

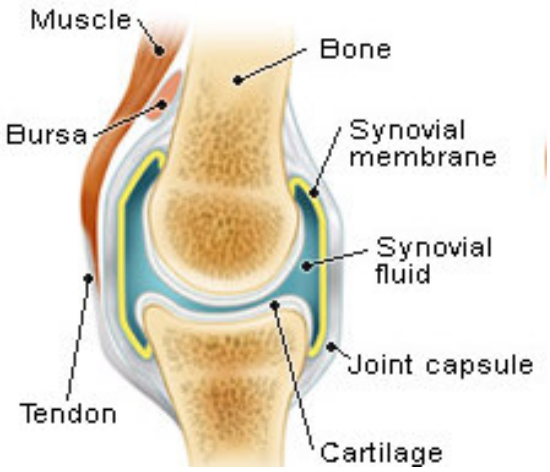
The symptoms associated with OA are significant functional impairment and signs and symptoms of inflammation like pain, stiffness, swelling and loss of mobility. The risk of developing OA increases with age and the risk factors include obesity, injury, previous surgery and overload to the joints [3]. RA is considered an autoimmune disease where the immune system attacks the bodies own cells and tissues. This results in a chronic articular disease where inflammation causes destruction of cartilage and bone [4]. Considering the different aetiology of arthritic conditions, inflammatory joint diseases represent a heterogeneous group of disorders that share features of deteriorated extra cellular matrices in articular cartilage and bone. Arthritis affects all structures within a joint, not only is articular cartilage lost, but bone remodelling occurs with capsular stretching and weakness of periarticular muscles [3].

Arthritis results in the release of inflammatory mediators in the synovial fluid and synovium that directly and indirectly influence cartilage homeostasis [5]. Recent studies show that chondrocytes in the cartilage actively participates in the disease process, and especially in the molecular signalling that occurs. Chondrocyte activity includes receptor signalling, production of matrix metalloproteinases (MMP), cytokines, and cell proliferation [5]. Studies of early RA show that cartilage destruction is not only a result of inflammation mediated by immune cells, but may in part be caused by the chondrocytes themselves; this is apparent in early cases of OA where cartilage destruction occurs without overt clinical signs of inflammation [6, 7]

1.1 Synovial Joints

Human joints are classified according to structure and function; how the bones are connected to each other and the degree of movement between the articular bones. Classified by structure humans have; fibrous joints connected by fibrous connective tissue, cartilaginous joints joined by cartilage, and synovial joints where the bone is not directly joined. Synovial joints are the most common and moveable joints in the human body and include; wrist, elbow, thumb, shoulder, hip and knee. In humans the knee joint supports nearly the entire body weight, thus being vulnerable to acute injury and arthritis [8].

The articular surfaces of synovial joints are covered by a thin layer of hyaline cartilage up to 3 mm thick. A viscous and clear synovial fluid lubricates the hyaline cartilage, supplying essential nutrients to the chondrocytes. A normal knee will have approximately 2 ml of synovial fluid that normally contains around 100 cells/mm^3, these cells are mostly lymphocytes and macrophages. Synovial joints are enclosed by a fibrous capsule which is lined by a thin synovial membrane. The synovial membrane comprises to cell types; modified macrophages (synoviocytes type A) and fibroblast-like cells (synovicytes type B), these cells synthesize and secrete hyaluronic acid and proteins of the synovial fluid. The fibrous capsule of the joint and the bone underlying the articular surface contains several sensory nerve endings. In contrast, the synovial membrane has no specialised sensory receptors and is therefore relatively insensitive to pain [8].

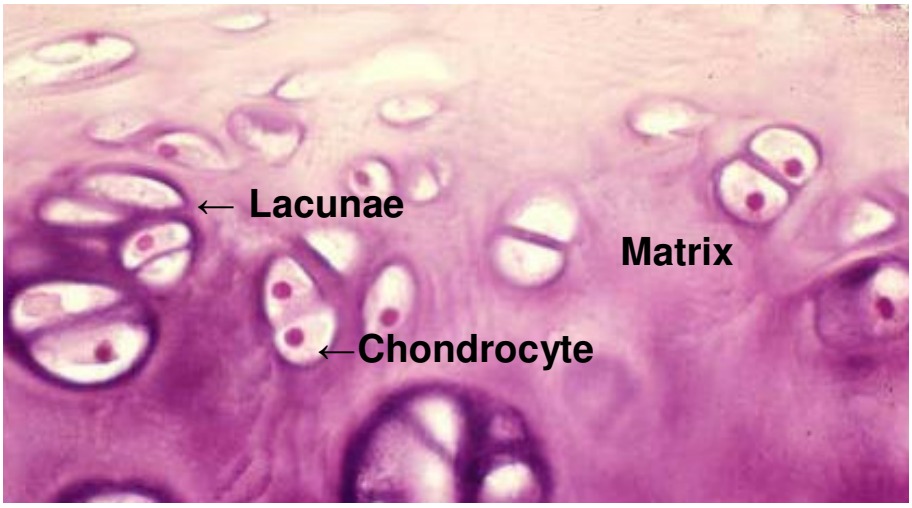


http://www.medicinenet.com/osteoarthritis/article.htm

Figure 1: Human synovial joint

1.2 Hyaline cartilage

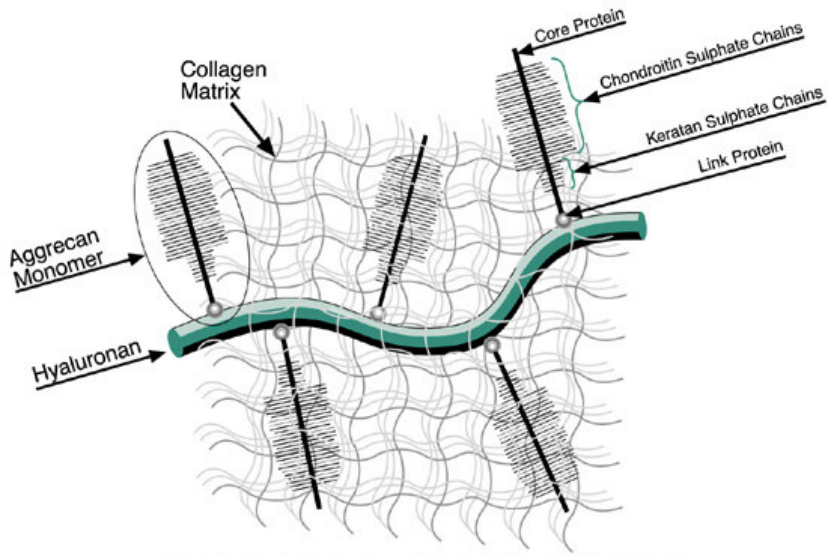
Hyaline cartilage is a firm and flexible connective tissue, and is specialised to absorb and resist compression. The tissue is supported by underlying bone and lubricated and nurtured by the synovial fluid. Nutrition and waits disposal has to be transported by diffusion through the synovial fluid due to the absence of blood vessels and lymphatic-drainage in the cartilage. The cells present in the cartilage are chondrocytes, and are contained in cavities in the matrix called “lacunae”, representing 1-5 % of the hyaline cartilage (Fig. 2).



http://www.meddean.luc.edu/lumen/MedEd/Histo/frames/h_frame9.html

Figure 2: Histology section of hyaline articular cartilage.

Chondrocytes synthesize and secrete macromolecules that constitute the cartilage matrix. Chondrocytes replenish the matrix as the macromolecules turn over, but their ability to remodel and repair the matrix is rather limited. The extracellular material consists of large hydrated proteoglycan aggregates, trapped within a matrix of type II collagen fibrils. The entrapped proteoglycans, mainly aggrecan, are composed of a core protein that forms a backbone, which glycosaminoglycan (GAG) chains are covalently attached to. These chains extend from the backbone in a brush-like structure that allows trapping of water molecules (Fig. 3). The GAGs are negatively charged and generate large osmotic swelling pressures that produce a hard compression-resistant substance. The three major GAGs found in cartilage are chondroitin sulphate, keratan sulphate and hyaluronan. Numerous chains of chondroitin sulphate and keratan sulphate linked to a core protein constitute the abundant proteoglycan aggrecan [9].



http://www.peprotech.com/content/focusarticles.htm?id=72

Figure 3: A schematic presentation of the extracellular martix of cartilage.

1.3 Inflammation

The immune system can be classified as innate (non-specific) and adaptive (specific). The innate system is the first line defence against pathogens and participants are cellular and non-cellular components like the complement system, epithelial cells, polymorphonuclear leukocytes (PMN), mononuclear cells, and conserved innate immune receptors like the Toll-like receptors (TLR). In the innate immune system, phagocytes and soluble factors unspecifically eliminate or neutralize pathogens [10]. The adaptive immune system involves building immunity over several days and is based on specificity and memory, characterized by antibody production and lymphocyte activation. Whenever the innate immune system is unsuccessful, the adaptive system will be initiated in the lymphoid organs, where mature dendritic cells (DCs) or macrophages present antigens to naive or memory T-cells [11].

The innate immune system initiates' acute inflammation in response to tissue injury and the usual outcome is successful resolution, destruction of invading microorganisms, prevention of spread, and promotion of subsequent damage repair. The acute inflammation is often characterized by the rapid influx of PMNs, typically neutrophils, followed by monocytes that mature into inflammatory macrophages that proliferate. Resolution of inflammation occurs when the neutrophils are eliminated and the tissue macrophages returns to normal pre-

inflammatory numbers and phenotypes [12]. When resolution fails, the acute inflammation may develop in to a chronic phase which may distort the tissues and cause permanent damage.

Inflammation involves interaction with the microvasculature, circulating blood cells and cells in affected tissues. These cells can synthesize and secrete a variety of soluble substances termed inflammatory mediators. Such mediators include cytokines and eicosanoids. Cytokines are peptides or glycoproteins that bind receptors on the surface of their target cells. Cytokines affects nabouring cells on the site of their production (paracrine action), on the cells that produce them (autocrine action), and they can affect multiple cell types, thus cytokines can be considered as signals alerting the immune system, conveying communication between the cells. Despite the beneficial role of inflammatory mediators in host defence, their sustained production can lead to serious, chronic pathological conditions, such as atherosclerosis and arthritis [13]. In chronic inflammation, the production of cytokines and proteases by infiltrating- and resident-cells, escape regulatory mechanisms. The imbalance between these mediators and their respective inhibitors is responsible for the persistence of chronic inflammatory conditions and may even be necessary for the initiation of chronic inflammation [14].

1.4 Joint inflammation and cartilage destruction

Joint inflammation in RA affects primarily the synovial lining of the joints, and the inflammatory process is characterized by proliferating synovial cells, increased vascularization, and infiltration of inflammatory cells [15]. In later stages there is an extension of the inflammatory tissue mass to the articular cartilage with progressive overgrowth of the aticular surface, and tongues of proliferating cells can be seen penetrating the extracellular matrix of the cartilage, commonly described as pannus. The RA synovium produces a variety of mediators capable of stimulating cartilage matrix destruction and states a connection between inflammation and the development of joint damage. However, joint destruction may progress in spite of attenuated inflammatory activity, and cartilage and bone erosion may develop in the absence of overt clinical signs of inflammation [7].

OA is not recognized as a classical inflammatory arthropathy [16] , and although there are debates regarding the essential role of synovial inflammation in OA, overexpression of pro-inflammatory mediators are common in early and late OA. In OA, synovial inflammation also

contributes to dysregulation of chondrocyte function, favouring imbalance between the catabolic and anabolic activities of the chondrocyte in remodelling the extracellular matrix [16].

Studies in humans and animals indicate that although the specific cellular mechanisms of cartilage destruction are not clarified, TNF-α, IL-1β and other pro-inflammatory cytokines appears to drive the process [4]. In RA the cartilage destruction can be explained by the release of proteinases by synovial cells capable of digesting the cartilage matrix components, along with the inflammatory cells that release inflammatory mediators in the synovial fluid. Yet, there is evidence of loss of proteoclygans and selective damage to type II collagen in the middle and deep zones of the cartilage [6] suggesting that the chondrocytes degrades its own matrix. Recent evidence suggest potential candidates responsible for cartilage destruction to be different MMPs which degrade collagens and proteoglycans [17]. MMPs are produced by both the synovium and chondrocytes in response to inflammatory cytokines, and it has been demonstrated that the pro-inflammatory cytokine IL-1β up-regulates the expression of several genes in chondrocytes, including MMP transcripts [17, 18]. Chondrocytes may not only participate in the destruction of the cartilage matrix by responding to pro-inflammatory cytokines, but can also contribute by producing pro-inflammatory cytokines themselves. This way, chondrocytes exert autocrine and paracrine effects increasing tissue catabolism and suppressing anabolic repair processes [5].

1.5 Chemokines

Antigen presenting cells (APCs) like the DCs and macrophages control the gateway between innate and adaptive immunity, thus APCs play a major role in chronic inflammatory diseases. APCs are activated by microorganisms through Toll-like receptors and release co-stimulatory molecules, cytokines and chemokines, thus being part of the innate immune system. The APCs further mounts the adaptive immune response by antigen presentation and T-cell activation [19]. APCs are attracted to infection- and inflammation-sites by a variety of factors, among which chemokines are the largest group so far [20]. Chemokines are generally classified as cytokines that control cell trafficking. In humans the chemokines are abundant, including more than 50 different types that can be separated into four families according to the location of their cysteine residues and their function [10]. The four chemokine groups are CC, C, CXC and CX3C, where C is a cysteine and X any amino acid residue. The largest

family consists of CC chemokines which attracts mononuclear cells to sites of chronic inflammation, monocyte chemoattractant protein 1 (MCP-1) is a classical example of a CC chemokine. The second largest family consists of the CXC chemokines which attract PMNs to sites of acute inflammation, an example here is interleukin-8 (CXCL8) [10]. Functionally, chemokines can be divided into inflammatory, haemostatic, and dual function. Inflammatory chemokines are induced by pathogens, cytokines and growth factors, and recruit effector leukocytes to sites of infection, inflammation, tissue injury and tumours [21].

The chemokines affect cells through surface bound chemokine receptors, these are seven transmembrane helical segments coupled to a G-protein that transduces intracellular signals. Chemokine receptors belongs to class A of the G-protein coupled receptor (GPCR) super family, and are rhodopsin-like receptors that span the membrane seven times and are coupled to heterotrimeric Gαβγ proteins [21]. Each chemokine receptor may have multiple ligands, thus several chemokines can bind different receptors and activate intracellular pathways with similar cellular responses [22]. The chemokine characteristics and the chemokine receptors involvement in leukocyte recruitment to inflammatory sites has rendered them attractive targets for therapeutic applications [23].

In the context of arthritis, synovial tissue and synovial fluid from RA patients contain increased concentrations of multiple chemokines, and the inflammatory cells that infiltrate the joint express respective chemokine receptors [24]. Several studies show increased levels of multiple chemokines in RA compared to OA, and this may reflect different inflammatory activity in the two conditions [25, 26]. The chemokine production in RA is mainly a product of macrophages and synoviocytes, however, studies show that chondrocytes can produce chemokines as well [22, 27]. Chemokines produced in the joint recruit leukocytes into the joint cavity and stimulate chondrocytes and synovial cells to release inflammatory mediators, including pro-inflammatory cytokines and MMPs which can lead to cartilage destruction [22].

1.6 G-protein coupled receptor- ChemR23

ChemR23 is a Gαi linked, G-protein coupled receptor, also called Chemokine-Like Receptor 1 (CMKLR1). ChemR23 exhibits homology to chemokine receptors although it is not classified within the chemokine receptor family [28]. Most chemoattractant molecules are characterized by a relatively poor selectivity, as they usually attract a diverse set of leukocytes. Within the leukocyte population, ChemR23 is rather specific, being expressed mainly in macrophages and plasmacytoid DCs, the two major classes of APCs [23]. Additionally, the receptor was identified in 2007 on $CD56^{low}CD16^{+}$ natural killer (NK) cells [29], and due to the receptors role in co-localization of NK celles, DCs and machrophages, both the receptor and its ligand represent attractive candidates for future drug development.

ChemR23`s natural ligand Chemerin, was identified in 2003 by Wittamer et al. where the authors described Chemerin as a potent chemoattractant factor for ChemR23 positive cells, thus attracting APCs [19]. Chemerin is initially synthesized and secreted as pro-Chemerin, an inactive form of the peptide. Chemerin is a potent ligand for ChemR23 when proteolytic removal of the last six amino acids at the C-terminal end occurs. Active Chemerin is present in plasma, serum and hemofiltrate, and the estimated concentration in plasma and serum in humans are 3.0 and 4.4 nM, respectively [30]. Chemerin is present in several tissues including liver, pancreas and lungs, and are abundant in inflammatory fluids [19]. According to Wittamer et al. activated neutrophils in the early phase of inflammation releases serine proteases cathepsin G and human leukocyte elastase, and these proteases mediate the conversion of pro-Chemerin into active Chemerin. Hence, the Chemerin may have a potential role as a link between innate and adaptive immunity by attracting APCs when activated by neutrophils [23]. Zabel et al. showed in 2005 that also serine proteases of the coagulation- and fibrinolytic-cascades activates Chemerin, thus implicating the involvement of Chemerin in tissue damage and bleeding as well as in the inflammatory cascade [31].

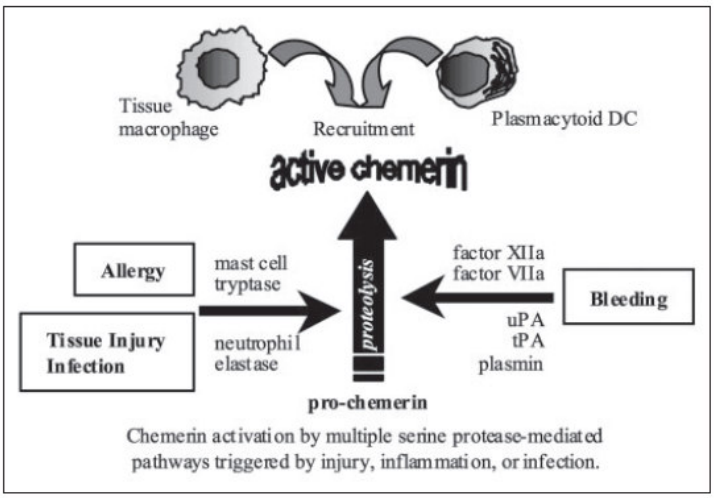


Figure 4: Chemerin activation by multiple serine protease-mediated pathways triggered by injury, inflammation or infection [31]

Chemerin was first described as a pro-inflammatory plasma peptide based on its ability to induce immune cell migration; however Cash et al. has in 2008 shown that murine Chemerin also can exhibit anti-inflammatory effects on macrophages. According to the latter authors, activated macrophages may be capable of converting Chemerin into a potent anti-inflammatory peptide by cysteine proteases calpains and cathepsin S. They show that designed Chemerin peptides can bind macrophages and inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL- 6, IL-12 and RANTES, and increase the production of anti-inflammatory cytokines like TGF-β and IL-10 [13]. Cash et al. thereby postulates that enzymatic proteolysis of pro-Chemerin can result in the generation of both activating and inhibitory peptides. Whereas serine proteases capable of producing activating peptides are released from activated neutrophils [23], cycteine proteases that generate inhibitory peptides, are released from activated elicited macrophages [13].

Several research groups have described a role of Chemerin in adipogenesis and adipocyte metabolism, and characterize Chemerin as a novel adipokine [32-34]. Their results contradict previous reports where ChemR23 is stated to be expressed only by macrophages, NK cells and plasmacytoid DCs. According to Goralski et al. Chemerin and its receptor are expressed in human adipocytes which indicate that white adipose tissue is both a source and a target for Chemerin [33]. Furthermore, it has been observed that adiposity associated with obesity

induces inflammation in adipose tissue which may correlate to the chemotactic ability of Chemerin (Fig. 5).

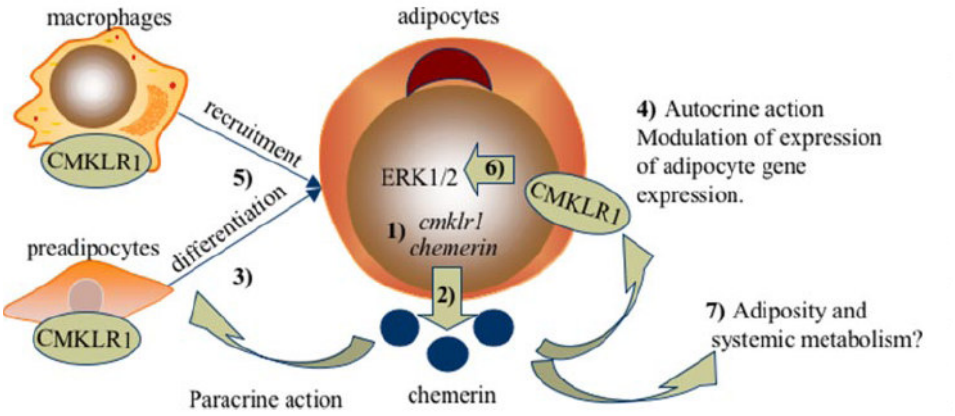


Figure 5: Secreted Chemerin may mediate recruitment of ChemR23 (CMKLR1)-expressing cells (e.g. machrophages and DCs) to adipose tissues [33].

1.7 Receptor signalling

As previously described, ChemR23 is a member of the seven-helix receptor family also called G-protein coupled receptor super family (serpentine receptors). Common for these receptors is the transfer of signals with the help of G-proteins to effector proteins that alters the concentration of ions and second messengers inside the cell (Fig. 6). The G-protein involved in ChemR23 signalling consists of three subunits (α, β and γ) where the α-subunit can bind GDP or GTP and has GTPase activity. The G-proteins can be divided into: stimulatory G-proteins (Gs), which activate adenylate cyclases or influence ion channels; inhibitory G-proteins (Gi), which inhibit adenylate cyclases; and the Gq proteins, which activates the effector enzyme phospholipase c. Binding of ligand to the 7-helix receptors alters the receptor confirmation in such a way that the G-protein can attach on the inside of the cell. This attachment causes the α-subunit to exchange bound GDP for GTP which releases the G protein from the receptor, and thereafter the G protein dissociates into a α-subunit and a βγ-unit. Both units bind other membrane proteins and alter their activity: ion channels can be opened or closed, and enzymes can be activated or inactivated [35].

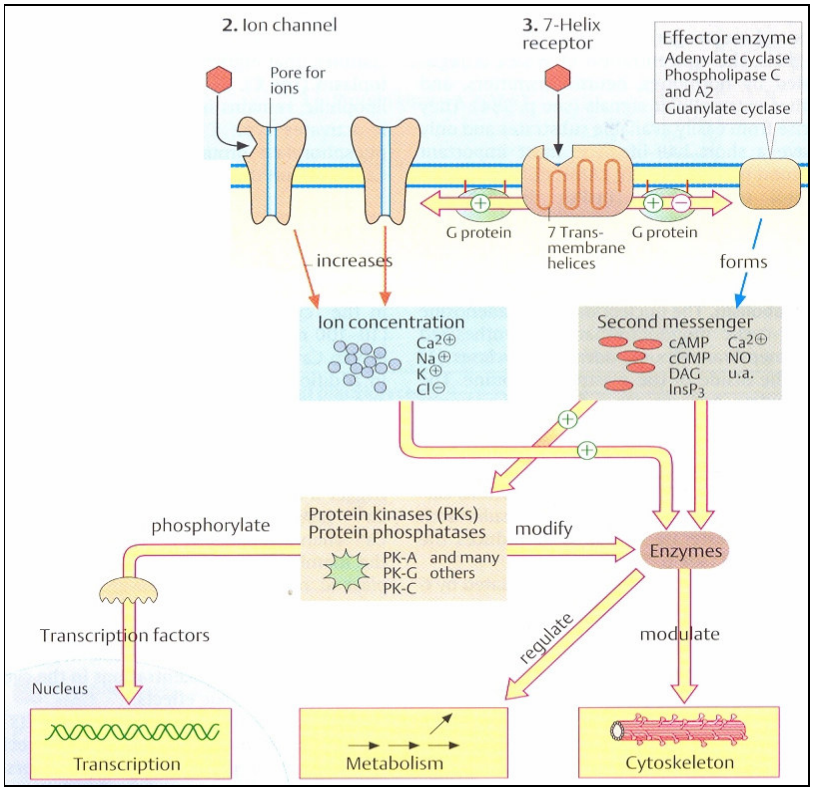


Figure 6: Schematic overview of 7-helix receptor signalling [35]

The ChemR23 is a Gi coupled receptor where the Gi proteins inhibit adenylate cyclase activity. Activation of the receptor on macrophages and DCs, results in intracellular calsium release, inhibition of cAMP accumulation, phosphorylation of p42-p44 MAP kinases (ERK 1/2) and elevated levels of the transcription factor NF-κB. In macrophages and DCs, this intracellular pathway creates a pro-inflammatory profile with the initiation of pro-inflammatory transcripts [19]. Zabel et al has newly identified an orphan receptor on mast cells, CCRL2, which binds Chemerin as well. However, this receptor does not conduct intracellular signalling when Chemerin binds, although local concentration of Chemerin is increased [36]. It might be that this receptor functions as a decoy receptor, neutralizing bioactive Chemerin by reducing its bioavailability.

2. Aim of study

The current medical treatment of joint disease is predominantly based on anti-inflammatory therapy where inflammatory cells and their mediators are targeted. Less attention has been given to the chondrocytes and their potential as a therapeutic target for preventing cartilage destruction. Marked levels of active Chemerin are present in inflammatory fluids, and also in synovial fluid from inflamed joints. Given recent evidence showing that chondrocytes participate in cartilage degradation, and the fact that active Chemerin is present in synovial fluid, we put forward a hypothesis that chondrocytes express ChemR23, and that Chemerin binds and activates the receptor to affect cartilage metabolism through MMP- and cytokine-production.

The specific aims of the study were:

1. To show that chondrocytes express ChemR23 receptor
2. To show that ligand binding of the receptor leads to activation of intracellular signalling mechanisms.
3. To show that Chemerin stimulate the chondrocytes to produce pro-inflammatory cytokines and MMPs.

3. Materials and methods

3.1 Acquisition of chondrocytes

Patients undergoing autologous chondrocyte transplantation (ACT) gave their informed consent for use of surplus cells from the transplantation programme. The patients, aged 25-45 suffered either from focal cartilage damage in knee joints or late stages of OA. Biopsies were taken from the articular surface at a low-weight bearing area of the joint and thereafter enzymatically digested [37]. The cells were serially passaged for 3 weeks to obtain a sufficient cell number and surplus cells were cryo-preserved for experiments.

3.2 Culture medium

The medium used for cultivating the chondrocytes was DME/F12 (Cat. No. 3-821-35, Imperial Laboratories Ltd. Andover, UK). The medium was a basal powder that was solved in 500 ml sterile water (Cat. No. 883314, Pharmacia, Stockholm, Sweden) and supplemented with 2.2 g/l NaHCO3 (Cat. No. 1.06329.1000, Merck), 62 µg/ml ascorbic acid (Cat. No.A-4034, Sigma, St.Louis, MO), and 50 µg/ml gentamycin (Cat. No. G-1264, Sigma). The mediums osmolality was adjusted to 290 nOsm/l using a cryoscopic osmometer (CryoStat, Gonotec GmbH, Berlin, Germany) and 280 µl H2SO4 (Merck, Darmstadt, Germany) was added. Finally the medium was sterile filtrated (0, 2 µM). 10 % autologous serum was added to the medium when cultivating the cells the first three weeks.

The chondrocytes were stored in medium DME/F12 supplemented with 5 % Cryoserv (Baxter Research Medcal, Irvine, CA, USA) and kept in liquid nitrogen at -180 ºC. At the experimental start the cells were thawed and the cryopreservative was removed by two consecutive washings. The ampoule contents were transferred to sterile tubes with warm medium supplemented with 10 % foetal calf serum (Cat. No. 14-830L, Lonza, Medprobe, Norway), and the pellet was resuspended in fresh medium after centrifugation at 200 g. Finally the cells was resuspended in 5 ml fresh medium after the second wash, and transferred to 250 ml culture vessels (Cat. No. 3108, Falcon, BD Bioscience, Stockholm, Sweden) kept in humidified air containing 5 % CO_2. Cultures were further expanded by trypsination (Cat. No T-3924, Sigma), and transferred to 500 ml culture vessels (Falcon). Experiments were performed when an appropriate cell number was achieved as judged by microscopy.

3.3 Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

Poly A mRNA from chondrocytes was extracted with Qiagen Direct mRNA kit (Merck Eurolab, Oslo, Norway). cDNA was synthesised by using SuperScript Preamplification System (Life Technologies Ltd., Paisley, UK) and was treated with 0.1 unit/l *Escherichia coli* RNase H at 37 ºC for 20 min.

PCR was performed in a 50 µl reaction mixture containing cDNA (derived from 0,5 g mRNA), 150 nM of each primer, master mix containing taq polymerase, dNTPs, MgCL and buffer (5 Prime MasterMix, Cat.no. PRME2200100, VWR, Oslo, Norway) and ultra pure distilled water (10977-035, Gibco, Invitrogen, Oslo, Norway). The PCR were performed at 94ºC for 5 min (first denaturation), then at 94ºC for 30 sec (denaturation), 55ºC for 30 sec (annealing) and 72ºC for 1 min (extension) for a total of 30 cycles with a 10 min final extension at 72ºC. All reactions were run using a Perkin-Elmer GeneAmpPCR system 2400 (Perkin-Elmer, Cambridge, UK)

Primers:

Nucleotide sequences of PCR primers were designed to detect domains of the human ChemR23 receptor:

Forward ChemR23 5´-TGG TCT ACA GCA TCG TC-3´

Revers ChemR23 5´-ATG GCT GGG GTA GGA AGA GT-3´

PCR product length 917-bp [38]

To test the quality of the mRNA, the presence of APRT-gene transcripts was assayed. With the primers used, contaminating DNA would generate an 800-bp fragment, while mRNA would generate a 300-bp fragment:

Forward APRT 5´- CCC GAG GCT TCC TCT TTG GC-3´

Reverse APRT 5´-CTC CCT GCC CTT AAG CGA GG-3´

PCR product length 300-bp [39]

Genomic DNA was acquired from isolated DNA, collected from patients analyzed for Leiden mutations.

PCR products were analyzed by polyacrylamid gel (Novex TBE gel 6 % Cat no. EC6265BOX, Invitrogen, Norway), stained with SYBR-safe DNA gel stain (Cat. No S33102, Invitrogen) and photographed under UV-light using a G-BOX (Syngene, Cambridge, UK).

3.4 Immunocytochemistry

Identification of the ChemR23 receptor on serially cultured chondrocytes was performed with polyclonal rabbit anti-human ChemR23 antibody (Cat. No.ab13172, Abcam, Cambridge, UK). Chondrocytes were grown on fibronectin coated chamber slides (Cat No. 154534, Nunc, Roskilde, Denmark) for 24 hours. The cultures were washed twice with PBS (phosphate buffered saline) and fixated for 2 hours in cold PBS containing 0.2 M sucrose and 4 % paraformaldehyde. After fixating the cells the slides were blocked for one hour with PBS containing 1 % BSA (Bovine Serum Albumine, Cat No.A2153-10G, Sigma-Aldrich, Sweden AB, Stockholm, Sweden). After blocking, the slides were washed twice with PBS and primary antibody diluted 1:250 was added. The slides were incubated in primary antibody overnight at 4ºC. Then the slides were washed three times in PBS and secondary antibodies were added to sections at dilution 1:200 (Alexa fluor 488 conjugated goat anti-rabbit IgG, Cat No. A-11008 and Alexa fluor 568 conjugated goat anti-rabbit IgG, Cat. No. A-11011, Invitrogen), and incubated for 1 hour in room temperature. Negative controls were arranged by omitting primary antibody in respective sections. The slides were mounted by adding Dapi-fluoromount-G (Cat. No. 0100-20, SouthernBiotec, Birmingham, US) and examined in a Zeiss axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) equipped with Nikon Coolpix 995 digital camera.

3.5 Immunohistochemistry

Sections of a cartilage biopsies were made to study whether ChemR23 was present in native tissue. Paraformaldehyde (4 %) containing 0.2 M sucrose in PBS was used as fixative, and after 48 hours the biopsy was embedded in paraffin and sectioned at 5 µm thickness onto poly-L-Lysine-coated slides (0,01 %, Sigma-Aldrich). Sections were deparafinized by xylene and graded alcohol washes, and immersed in distilled water. Thereafter sections were incubated in PBS containing 1 % bovine serum albumin for 30 min followed by incubation with monoclonal mouse anti-human chemerin antibody diluted 1:100 (Cat. No. MAB362, R&D systems, UK) overnight at 4ºC. After rinsing in PBS, sections were incubated for 45 min with secondary antibody which were rabbit anti-mouse antibody conjugated with

horseradish peroxidase (HRP). SuperPicTure Polymer detection kit was used (Zymed Laboratories, San Francisco, CA, USA). A matched isotype was used as a control for nonspesific background staining.

3.6 Western blotting of phospho-p44/42 MAPK (ERK1/2) and phospho-Akt

Intracellular signal transduction triggered by Chemerin was investigated by immunoblotting of phosphorylated p44/42 (Thr202/Tyr204) and phosporylated Akt (Ser473) by using phospho-Erk1/2 pathway sampler kit (Cat. No. 9911, Cell Signaling Technology, US). Antibodies raised in rabbit towards phospho-p44/42 (Thr202/Tyr204) and phospho-Akt (Ser473) were used to detect ChemR23 mediated phosphorylation of the respective molecules.

Cell cultures were arranged in three main groups; one group was challenged with recombinant Chemerin (Cat. No. 2324-CM, R&D systems, UK) and incubated in different time intervals. One group represented the control where only PBS was added, and the third was incubated with MEK1/2 inhibitor (Cat.No 9900, Invitrogen) and Chemerin. MEK 1/2 inhibitor was added to the cell culture one hour prior to stimulation with Chemerin. 0.5 $x10^6$ cells were added to each well in a 6 multiwell plate (Cat. No. 3046, Falcon) and grown in culture medium with 10 % FCS for 24 hours. Subsequently the cells were washed twice in PBS and incubated for 24 hours in culture medium without FCS. The next day cultures were washed twice and challenged with 10 nM chemerin for 1 min, 2.5 min, 5 min and 10 min. The wells were washed with PBS and harvested directly in 150 µl SDS-buffer containing NuPAGE LDS sample buffer (Cat. No. NP0007, Invitrogen), NuPAGE Reducing agent (Cat. No. NP 0004, Invitrogen), phosphatase inhibitor (Cat. No. 78420, Thermo Scientific, Rockford, US), protease inhibitor (Cat.No. 04693124001, Roche, Indianapolis, US), and distilled water.

The samples were heated to 100ºC for 5 min. 15 µl of each extract including 15 µl of a prestained protein marker (Cat. No. 7720, Cell Signaling Technology, US) to control the efficacy of the electrophoresis, and 10 µl of a biotinylated protein ladder (Cat. No. 7727, Cell Signaling Technology, US) to assess molecular weights (kDa) of proteins, was separated by electrophoresis in NuPAGE Mes SDS running buffer (Cat. No. NP 0002, Invitrogen) at 200V (constant), using 100-125 mA per gel (NuPAGE 4-12 % BIS-tris gels, Cat. No. NP0323, Invitrogen) for 35 minutes. Electroblotting was performed by electrontransfer onto PVDF-

membranes (Cat. No. LC2005, Invitrogen) in NuPAGE transfer buffer (Cat. No. NP0006, Invitrogen) with 10 % methanol, at 30V (constant), using 170 mA per gel transfer for 1 hour. After electroblotting the membranes were blocked with 5 % non-fat dry milk/0,1 % Tween 20 for 1 hour at room temperature. Thereafter the membranes were incubated in primary antibodies overnight at 4ºC in 5 % BSA/0,1 % Tween 20. The phospho-p44/42(Thr202/Tyr204) antibody was used in a 1:2000 dilution, while the phospho-Akt(Ser473) antibody was diluted 1:1000. Then the membranes were washed in wash buffer and incubated with HRP (Horseradish preoxidase)-conjugated goat anti-rabbit IgG (Cat.No 7074, Cell Signaling Technology) and HRP-conjugated anti-biotin antibody (Cat. No. 7075, Cell Signaling Technology) for 1 hour in room temperature. Finally the proteins were detected by adding substrate containing Lumiglo reagent and Peroxide (Cat. No. 7003, Cell Signaling Technology) and developed with Fujifilm LAS-3000.

3.7 Cytokine measurement

Chondrocytes ($0.5x10^6$ cells) were added to three wells in a 6 well multiplate (Cat. No. 3046, Falcon) and incubated for 24 hour in culture medium with 10 % FCS. Thereafter the cells were washed twice with PBS and incubated 24 hours in culture medium with 0.1 % FCS. Thereafter the wells were washed twice and one well was added 1 ml of medium with 10 nM Chemerin, the second was added 1 ml medium with 100 nM Chemerin, and the third was added medium with PBS to represent the control. All cultures were incubated for 24 hours, before the medium supernatant was aliqouted and frozen in -70ºC for later experiment.

One ampule of each medium supernatant was thawed on ice, and the levels of the cytokines TNF-α, IL-1β, IL-6 and IL-8 was measured using a 4-plex cytokine assay (Cat No. X50053UVBS, Bio-Rad, Hercules, CA, USA) using the analyser Bio-plex 200 system (Bio-Rad,). The samples were run in a 1:4 dilution in duplicates.

3.8 MMP measurement

Chondrocytes ($0.5x10^6$ cells) were added to three wells in a 6 well multiplate (Cat. No. 3046, Falcon) and incubated for 24 hour in culture medium with 10 % FCS. Thereafter the cells were washed twice with PBS and incubated 24 hours in culture medium with 0.1 % FCS. Thereafter the wells were washed twice and one well was added 1 ml of medium with 10 nM Chemerin, the second was added 1 ml medium with 100 nM Chemerin, and the third was

added medium with PBS to represent the control. All cultures were incubated for 48 hours, before the medium supernatant was aliqouted and frozen in -70ºC for later experiment.

One ampoule of each medium supernatant was thawed on ice, and the levels of the MMPs MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13 was measured using a MMP-panel assay (Cat. No. LMP000, LMP901, LMP902, LMP513, LMP907, LMP908, LMP911, LMP919, LMP511, R&D) using the analyser Bio-plex 200 system (Bio-Rad,). The samples were run in a 1:4 dilution in duplicates.

4. Results

4.1 RT-PCR

Cultured cells from two individuals were analysed separately for transcripts for the Chemerin receptor ChemR23. The cDNA detected by gel electrophoresis show that the mRNA corresponding to the 917 bp transcript of the ChemR23 was detected in both cell cultures (Fig. 7, lane 5 and 6). The APRT primers gave two bands with 300 bp length which indicated no contamination of genomic DNA in the two samples (Lane 2 and 3). Both negative controls (Lane 4 and 7) were negative. This result demonstrates that cultured chondrocytes produce transcripts for ChemR23.

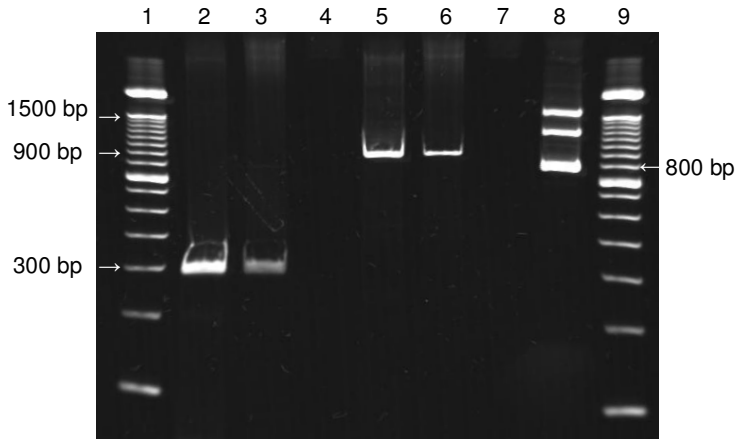


Figure 7: RT-PCR analysis of the expression of Chemerin receptor ChemR23 in cultured human articular chondrocytes. Lane 2 and 3: APRT primers were run with cDNA from two different cell cultures. The 300 bp bands show that the APRT transcript is generated from mRNA. Lane 4: APRT primers + water. Lane 5 and 6: ChemR23 primers were run with cDNA from two different individuals. The 917 bp bands indicate transcripts for the Chemerin receptor. Lane 7: ChemR23 primers + water. Lane 8: APRT primers were run with genomic DNA to show the 800 bp transcript that would have been visible if contamination with DNA had occurred.

4.2 Immunocytochemistry

Micrographs of cultivated chondrocytes showed a positive staining in presence of primary antibody compared to the unstained controls (Fig. 8). We used one primary antibody and two different secondary Alexa fluor conjugated antibodies to visualize the receptor, and both micrographs showed that the Chemerin receptor was expressed by serially cultured chondrocytes.

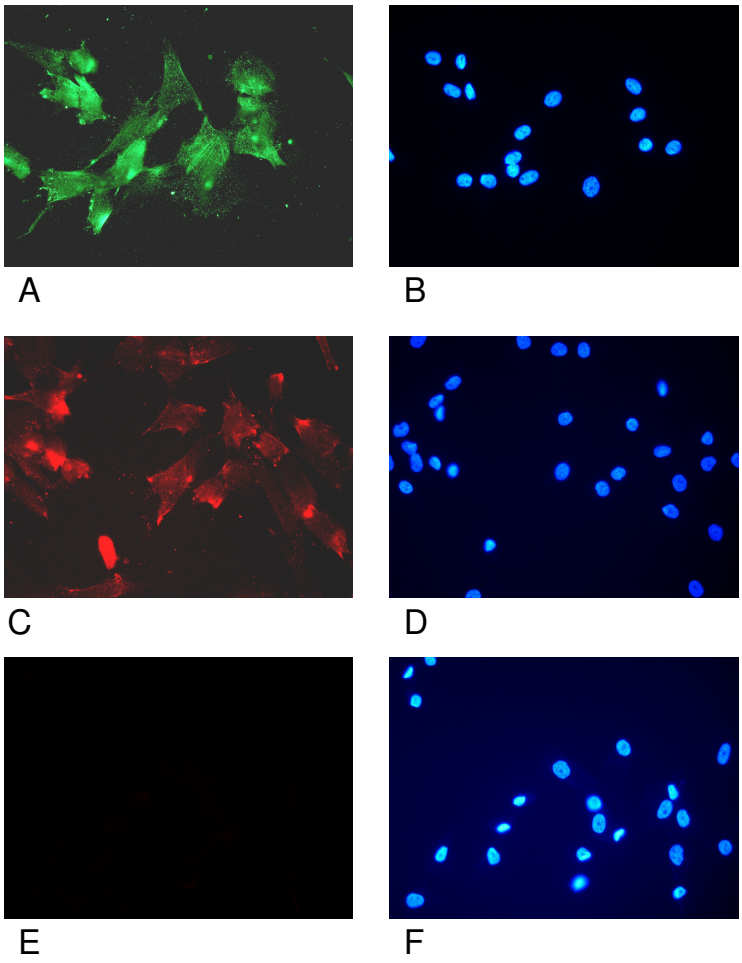


Figure 8: Cultured human articular chondrocytes stained with polyclonal rabbit anti-human ChemR23. Micrograph A show positively stained chondrocytes using antibody conjugated with Alexa fluor dye 488. Micrographs B represent the same picture but here the nuclei of the chondrocytes are visualized by Dapi dye. Micrograph C and D show positively stained chondrocytes using Alexa fluor 568 (C), and visualized nuclei by Dapi (D). Micrographs E and F show negative controls, i.e. primary antibody was omitted (E) and the nuclei of the chondrocytes (F).

4.2 Immunohistochemistry

Sections of cartilage tissue showed positively stained chondrocytes in the presence of primary antibody compared to the unstained isotype control (Fig. 9).

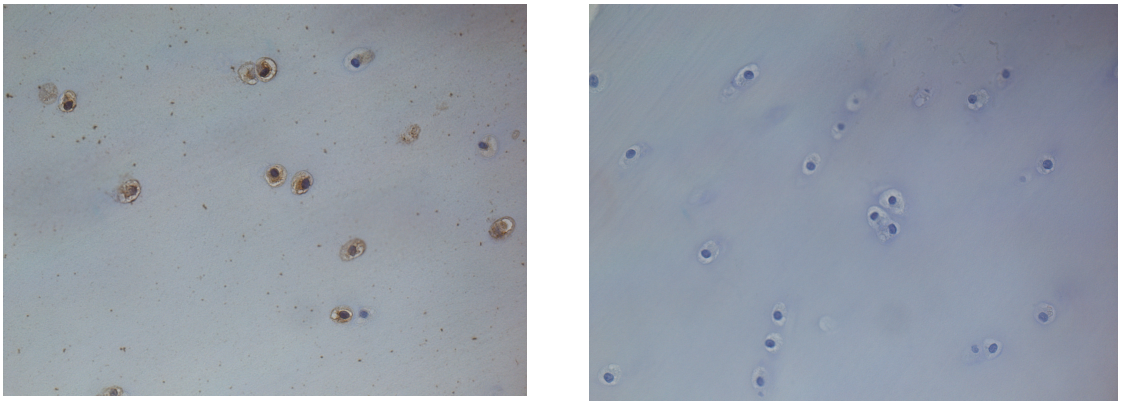


Figure 9: Sections of human articular cartilage tissue (400X) stained with monoclonal mouse anti-human ChemR23. Micrograph A show positively stained chondrocytes (brown staining). Micrograph B show unstained chondrocytes and represent the negative control where the primary antibody was replaced with a matched IgG isotype antibody to check for unspecific binding.

4.3 Western blotting for phospho-p44/42 (ERK 1/2) and phospho-Akt

In Western blot studies of cultured chondrocytes challenged with 10 nM Chemerin for 1 min, 2.5 min, 5 min and 10 min, bands occurred where anti-phospho-p44/42(Thr202/Tyr204) were used (Fig.10). In lane 1 representing unstimulated cells, a band also occurred and indicated a background of phosphorylated p44/42. The bands occurring after stimulating with Chemerin for 5 and 10 minutes were denser, showing a higher activation of the p44/42 MAPK as a result of ChemR23 activation by Chemerin. In lane 2, the MEK1/2 pathway was inhibited and this could be seen by weak bands, showing reduction of phosphorylated p44/42. These results demonstrate that Chemerin binds ChemR23 and activates the p44/42 MAPK pathway in chondrocytes.

In a paralleled experiment, detecting phospho-Akt (Ser473) by Western blot, bands occurred where anti-phospho-Akt (Ser473) was used (Fig.11). In unstimulated cells (Lane 1), there was a weak band present, indicating a low background level of phospho-Akt. The bands occurring after stimulating with Chemerin shows that phosho-Akt levels increases from the unstimulated sample to 5 minute of challenge with Chemerin, and then decreases after 10 minutes of challenge. These results demonstrate that Chemerin activation of ChemR23 increases phosporylation of Akt which may induce activation of MEK1/2 (Fig. 12). This correlates with the increase in phospho-p44/42 at 5 and 10 minutes (Fig. 10, lane 5 and 6).

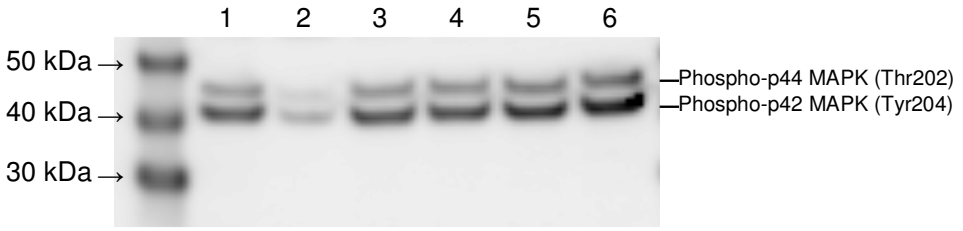


Figure 10: Western blotting of phosphorylated p44/42 (Thr202/Tyr204)
Following starvation for 24 hours, human chondrocytes were challenged for 1 min (Lane 3), 2.5 min (Lane 4), 5 min (Lane 5) and 10 minutes (Lane 6) with 10 nM recombinant Chemerin. For the visualization of phospho-p44/42, rabbit anti-human-phospho-p44/42 was used. Bands can be seen in lane 3, 4, 5 and 6 and the density increases after 5 and 10 minutes. Lane 1 represents the control where no Chemerin was added. The band observed in this lane indicates background phosphorylation of p44/42. Lane 2 represents the sample extract where the MEK 1/2 kinase inhibitor was added 1 hour prior to 5 minutes challenge with 10 nM Chemerin.

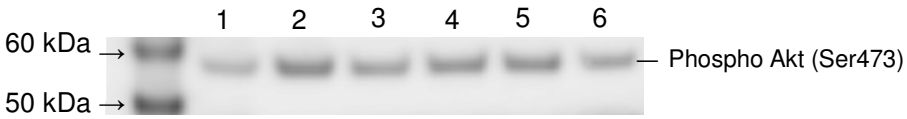
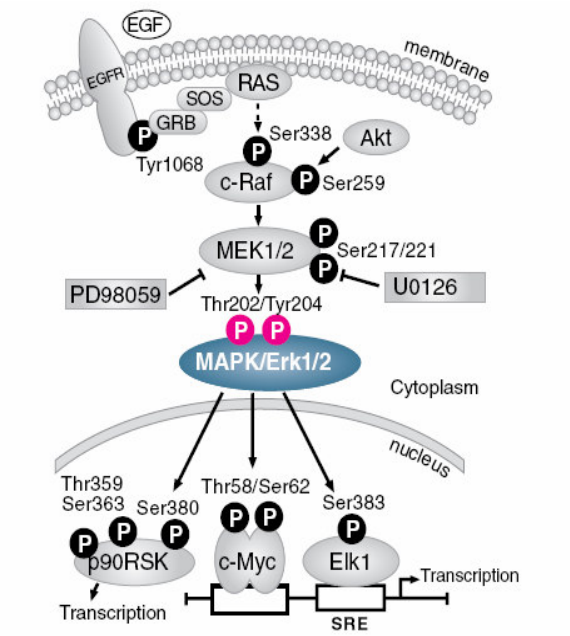


Figure 11: Western blotting of phosphorylated Akt (Ser473)

For the visualization of phospho-Akt, rabbit-anti-human-phospho-Akt (Ser473) was used. Bands in lane 3-6 represent cell cultures challenged with 10 nM chemerin for 1, 2.5, 5 and 10 minutes and indicate that the phosphorylation of Akt increases with increased stimulation time and decreases around 10 minutes of stimulation. Lane 1 represents the negative control and shows a low background phosphorylation of Akt. Lane 2 represent the culture where the MEK 1/2 was inhibited, and shows that inhibition of MEK 1/2 pathway does not effect the phosphorylation of Akt.



Ref: http://www.cellsignal.com/pdf/9911.pdf

Figure 12: Overview of the MAPK signalling pathway

Phosphorylated Akt can activate the MEK 1/2 pathway by phosphorylatin c-Raf. MEK 1/2 activates the MAP-kinases p44/42 (Erk 1/2) by phosphorylation of the amino acids threonine 202 and tyrosine 204.

4.4 Cytokine measurement

The measurement of cytokines in supernatants from chondrocytes challenged with Chemerin, showed an increased concentration of TNF-α, IL-1β, IL-6 and IL-8 compared to the unstimulated control (Table 1). IL-6 and IL-8 were markedly increased compared to IL-1β and TNF-α, and the cytokine levels were increased in a dose-dependent manner. These results demonstrate that Chemerin stimulates cultivated chondrocytes to produce pro-inflammatory cytokines.

Table 1: Cytokine levels in supernatants from three individual chondrocyte cultures challenged with chemerin *in vitro* for 24 hours.

Cytokine		Unstimulated	10 nM Chemerin	100 nM Chemerin
TNF-α	Patient 1	710	727	880
pg/ml	2	747	741	840
	3	154	222	245
IL-1β	Patient 1	147	154	187
pg/ml	2	158	156	179
	3	30	44	48
IL-6	Patient 1	2572	29352	41010
pg/ml	2	28410	32429	34264
	3	4010	5617	6707
IL-8	Patient 1	19937	20715	53255
pg/ml	2	31849	32191	49448
	3	2700	4050	4964

4.5 MMP measurement

The measurement of eight different MMPs in supernatants from three individual chondrocyte cultures challenged with Chemerin, showed an increased concentration of MMP-2, MMP-3 and MMP-13 compared to the unstimulated control (Table. 2). A marked increase of all three MMPs was observed for two of the patient samples, while a modest increase of the three MMPs was observed for the third patient. These results show that Chemerin induces the production of MMP-2, MMP-3 and MMP-13 in chondrocytes.

Table 2: MMP levels in supernatants from three individual chondrocyte cultures challenged with chemerin *in vitro* for 48 hours.

		Unstimulated	10 nM Chemerin	100 nM Chemerin
MMP-1	**Patient 1**	64857	63947	72501
pg/ml	**2**	73125	68601	74293
	3	45797	54196	71109
MMP-2	**Patient 1**	182813	164869	270921
pg/ml	**2**	91327	92796	109885
	3	97634	99084	320164
MMP-3	**Patient 1**	477649	456698	899049
pg/ml	**2**	506769	432008	598323
	3	50442	54571	204657
MMP-7	**Patient 1**	112	97	162
pg/ml	**2**	295	368	474
	3	107	115	156
MMP-8	**Patient 1**	109	81	81
pg/ml	**2**	702	702	969
	3	43	29	146
MMP-9	**Patient 1**	84	94	94
pg/ml	**2**	953	795	866
	3	68	85	70
MMP-12	**Patient 1**	<15	<15	<15
pg/ml	**2**	<15	<15	<15
	3	<15	<15	<15
MMP-13	**Patient 1**	8391	7828	19276
pg/ml	**2**	927	837	1003
	3	<84	<84	4503

5.0 Discussion

Rheumatic diseases are abundant and a large group of the population is affected. Rheumatoid Arthritis occurs in about 1 % of the population [22] and in the US, 70 % of the population between the age 55-78 have evidence of OA [2]. Considering the increasing lifespan of humans, arthritis will be a growing public health problem, involving large costs for the society [40]. To days treatment of arthritis is mainly based on anti-inflammatory therapy, pain relief and surgery in cases where the joint destruction is comprehensive. A lot of effort has been taken to understand the inflammatory process in joint diseases, and a better understanding has led to the development of several drugs against pro-inflammatory cytokines, like TNF-α blockers, with varying effects [41]. Although medication dampens symptoms, they do not necessarily prevent the disease progression [4]. It has been shown that arthritis may develop many years before any symptoms occur, but the lack of diagnostic tools makes the possibility for preventive treatment difficult [42, 43]. Recent findings have shifted the attention to the chondrocytes, and their role in the deterioration of cartilage matrix. It is clear that the chondrocytes are not completely isolated in the cartilage matrix, but actively participates in cell signalling in the joint [5]. There is evidence that the chondrocytes themselves are partly responsible for the deterioration of cartilage matrix, and the fact that cartilage destruction may occur without clinical signs of inflammation renders them a target for preventive treatment [7].

Cartilage destruction is mainly a result of imbalance in the cartilage metabolism. The features are often a shift in the balance between degrading proteases (MMPs) and the inhibitors of these proteases, Tissue Inhibitor of Metalloproteinases (TIMPs). This can be caused by pro-inflammatory mediators or by increased apoptosis/necrosis of chondrocytes which results in impaired matrix maintenance [44]. Our research group has previously demonstrated the presence of soluble TRAIL (TNF Related Apoptosis Inducing Ligand) in synovial fluid from inflamed joints, with levels up to150 pg/ml (unpublished data). We have further identified the TRAIL receptors DR4, DR5 and DCR2 on cultivated chondrocytes [45]. Soluble TRAIL binds DR4 and DR5 and induces apoptosis in chondrocytes. In 2002 we showed that chondrocytes express functional leptin receptors and that the activation by leptin induces cell proliferation and promotes matrix synthesis [39]. Results from several other research groups support our findings and the fact that chondrocytes can produce and bind a variety of cytokines and chemokines [1, 25, 27].

Previous results show that chondrocytes express several immune receptors, including class II histocompatibility antigens (MHC-II), which indicates immunregulatory activity by chondrocytes [46]. The present results further emphasize the role of chondrocytes in inflammatory diseases, as we here show that chondrocytes express Chemerin receptors. This is evident by the findings that chondrocytes produce ChemR23 transcripts, and that the receptor is present on chondrocytes in native cartilage and *in vitro*. The receptor binds recombinant Chemerin to elicit intracellular signalling leading to phosphporylation of ERK 1/2 and Akt, both being central signal transduction pathways for various inflammatory and metabolic responses [47, 48]. By stimulating cultured chondrocytes with Chemerin we show that ChemR23 conveys pro-inflammatory signalling in chondrocytes by stimulating the production of the pro-inflammatory cytokines IL-8 (Interleukin 8) and IL-6 (Interleukin 6), and affects cartilage metabolism by inducing the production of MMPs. Furthermore, it appears that Chemerin can exhibit dual effects in inflamed joints by attracting cells of the immune system to the inflamed joints [19], and at the same time bind to ChemR23 on chondrocytes and initiate a pro-inflammatory cytokine production. Our results show similarities with the discovery of the Chemerin receptors on adipocytes, and the ability of Chemerin to induce inflammation in adipose tissue [32, 33]. Hence, it can be suggested that Chemerin and ChemR23 have a role in the initiation of inflammation in cartilage tissue as well.

In addition to a marked elevation in IL-6 and IL-8 production, stimulation of chondrocytes with recombinant Chemerin also resulted in a modest increase of TNF-α and IL-1β. These results are consistent with reports showing elevated levels of these cytokines in adiponectin stimulated chondrocytes [1], and in synovial fluid from RA patients [49]. Several clinical observations have suggested a possible role for cartilage as a primary source of pro-inflammatory stimuli, as inflammatory responses often develops rapidly after traumatic cartilage injury [27]. Our results may portray a role of ChemR23 in the initiation of inflammation by chondrocytes as IL-8 is a potent chemokine witch exerts chemotacic activity towards neutrophils [50]. By this way, activation of the ChemR23 on chondrocytes indirectly attracts neutrophils by initiating the production of IL-8. This process may have implications in the development of chronic inflammation in joint disease.

Elevated production of IL-6 in supernatants from Chemerin stimulated chondrocytes was also observed, and Guerne et al. have previously showed that chondrocytes produce IL- 6 in response to physiologic and inflammatory stimuli, and that IL-6 may serve as a mediator to coordinate responses to cartilage injury [51]. IL-6 modulates the growth and differentiation of B- [52] and T-lymphocytes [53], and our findings may indicate that ChemR23 signalling can contribute to the activation of B- and T-cells in joint inflammation through IL-6.

IL-1β and TNF-α are characterized as the main drivers of cartilage and bone erosion [4]. Although we did not observe any significant elevation of these two cytokines, it might be that these mediators are produced mainly by immune cells and synovial cells as a result of inflammatory stimuli. These mediators may bind receptors on chondrocytes and stimulate the MMP production, thus affecting cartilage metabolism indirectly [54]. Furthermore, despite the modest increase in IL-1β and TNF-α , these levels may be sufficient to have a significant effect in the inflammatory process. Reports show that these cytokines together have strong synergistic effects at low concentrations [4]. TNF-α drives the acute inflammation, whereas IL-1 has a role in sustaining both inflammation and cartilage erosion [55]. Our results show that Chemerin stimulates TNF- α and IL-1β production by chondrocytes, which can initiate inflammation in joint diseases.

In arthritis, excessive degradation of cartilage matrix components by proteinases is main features of the destructive process. Depletion of proteoglycans from articular cartilage is a common initial change in joint disease with subsequent degradation of the collagen fibrils [56]. Our results show an increase in the production of the matrix metallo proteinases MMP-2, MMP-3 and MMP-13 in supernatants from Chemerin stimulated chondrocytes. These results are consistent with previously reports showing production of the these MMPs by chondrocytes *in vitro* [57-61]. The three MMPs we observed in the present study, all cleave the major proteoglycan aggrecan at the Asn^{373} –Phe^{342} bond (the MMP site), and the two major aggrecan fragments from cleavage of the respective sites, are detected in the joint fluid from patients with various arthritic diseases [62]. MMP-13 also selectively digests type II collagen which becomes denatured into gelatine. Gelatine is further digested into smaller peptides by gelatinases (MMP-2 and MMP-9) [63, 64].These observations supports our idea that ChemR23 plays a pivotal role in cartilage destruction, and our results show that Chemerin stimulates the production of MMPs in chondrocytes, and thus may portray a connection between inflammation initiation and cartilage deterioration.

According to earlier reports, the production of IL-8, IL-6 and MMPs in chondrocytes are assigned to the action of TNF-α and IL-1β [17, 27, 51, 65, 66]. The altered levels of IL-6, IL-8, MMP-2, MMP-3 and MMP-13 in our experiments, appears to be ChemR23-mediated. However, Chemerin may have induced a rapid increase of TNF-α and IL-1β at an early phase after stimulation, and the levels of IL-6, IL-8 and MMPs may be delayed products from autocrine actions on chondrocytes by TNF-α and IL-1β. Considering the 24 and 48 hours of Chemerin challenging, the cytokines may have peaked early during cultivation and thereafter been degraded or internalized. The different levels of cytokines and MMPs observed between the three individuals may reflect varying activation status in the cells during the cell preparations. Still, all the cultures showed an increase in cytokine/MMP quantity when challenged with Chemerin.

The source of Chemerin in synovial fluid is not yet identified, but Chemerin may enter the synovial fluid from the circulation through the capillaries surrounding the joint. Another possibility is that chondrocytes produce pro-Chemerin themselves. Considering how cytokines can act on their producer cells in an autocrine fashion, it may be that chondrocytes secrete pro-Chemerin which is then activated by neutrophil-derived proteases in the synovial fluid during inflammation.

As previously described, chemokines control immune cell trafficking, and infiltration of leukocytes is a hallmark of many autoimmune and chronic inflammatory diseases. For this reason approaches to prevent cell recruitment to inflamed tissues may be exploited, like preventing the effect of chemotactic mediators such as chemokines. Because cytokines mediate the production of chemokines, blocking the effect of chemokines has been done indirectly by blocking the biological effects of cytokines. This has been demonstrated by anti-TNF-α antibodies such as Remicade® and Humira®, souble TNF-α receptors such as Enbrel®, IL-1 receptor anatagonist Kineret®, anti-IL-6 receptor MRA®, and the anti-IL-15 antibody HuMax-IL-15®. Since cytokine and cytokine receptor reactions are complex, targeting specific actions has proven to be difficult and more attention is now given to chemokines that bind GPCRs. Several drugs currently marketed are either agonists or antagonists of GPCRs, i.e. these receptors have proven to be potent targets [21].

Developing antagonists against chemokine receptors has not yet been successful and this may be explained by the muliple functions of chemokines; a particular chemokine may bind several chemokine receptors and many chemokines may bind a single chemokine receptor. These features are characterized by different affinities between receptor and ligand, and activation of different signalling pathways [21]. A chemokine may act either as an agonist or an antagonist, depending on the receptor it binds [67, 68]. An example is the chemokine CXCL11, which is an agonist of a receptor mainly expressed on T helper type 1 (Th1) cells, while it acts as an antagonist on a receptor expressed on T helper type 2 (Th2) cells. This way the chemokine may attract Th1 cells to sites of injury, and simultaneously suppress the trafficking of Th2 cells to the same site. Despite the difficulties in developing antagonists to chemokine receptors, there is still intense activity in the field, and a number of agents are currently in development [69, 70].

Studies of inflammation has confirmed that the resolution process is an active process involving a pro-resolving signalling network [71, 72]. By measuring inflammatory exudates Serhan and colleagues have described lipid mediators that exhibit anti-inflammatory properties [73, 74]. These resolution-phase mediators use omega-3 polyunsaturated fatty acids as precursors to generate agonists for promoting resolution by reducing further recruitment of leukocytes. The omega-3 polyunsaturated fatty acids eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), are independently converted to resolvins and protectins [72]. Representatives of each family of lipid mediators, lipoxins from aracidonic acid (AA), resolvin E1 from EPA and neuroprotectin D1 from DHA, act at different steps in defined resolution indices, and are anti-inflammatory when given *in vivo* [71]. One member of the resolvin family, RvE1, is an aspirin triggered lipid mediator. Aspirin acetylates cyclooxygenase-2 (COX-2) and through the action of acetylated COX-2 and lipoxygenase (LO), EPA is converted into RvE1 through cell to cell interactions [73]. RvE1 has proven to be a strong anti-inflammatory and pro-resolving mediator, and reduces polymorphonuclear leukocyte (PMN) transendothelial migration and release of superoxide in the nanomolar concentration range [73, 75] These actions are also demonstrated *in vivo*, where RvE1 blocks PMN infiltration in peritonitis [76] and in inflamed colon tissue during colitis in mouse [77]. RvE1 actions on PMNs can be explained by the binding of RvE1 to the leukotriene B4 receptor (BLT1). RvE1 interacts with BLT1 as a partial agonist serving as a local damper of leukotriene B4 (LTB4)-BLT1 signals in PMNs, thus inhibiting PMN migration [76].

Arita et al. showed in 2005 that RvE1 binds both the LTB4 receptor, BLT1 and the Chemerin receptor, ChemR23 [76]. This way RvE1 attenuates APC functions by targeting DC migration and by reducing IL-12 production through ChemR23. Arita et al. further showed that RvE1 bound and activated ChemR23 to block TNF-a signalling, thus RvE1 mediate counter regulatory actions against ChemR23 to promote resolution. Compared to our result showing the expression of ChemR23 on chondrocytes, RvE1 may exibit similar actions on chondrocytes, making RvE1 a potential antagonist to Chemerin actions. Considering RvE1 actions on leukocytes it would be interesting to study whether RvE1 could both attenuate leukocyte recruitment into inflamed joints and at the same time prevent pro-inflammatory signalling in chondrocytes, thus preventing cartilage degradation. These studies are currently in progress in our research group.

Conclusion

The results presented in this thesis demonstrate the presence of a receptor previously not described on human articular chondrocytes, ChemR23. This receptor transduces intracellular signals through MAP kinases and Akt signalling pathways when challenged with Chemerin *in vitro*. It appears that activation of the receptor conveys pro-inflammatory signalling based on the findings demonstrating an increased pro-inflammatory cytokine production and MMP secretion. These results suggest that ChemR23 may have a pivotal role in cartilage destruction and the initiation and persistence of joint inflammation. Blocking ChemR23 from binding Chemerin may reduce inflammation in the joint. The receptors pro-inflammatory action renders it a potential therapeutic target and its ability to bind RvE1 renders ChemR23 to be a promising candidate for pro-resolution signalling. However, further studies are needed to confirm ChemR23s role in joint inflammation, and the applicability of its antagonists in human medicine.

References

References

1 Lago R, Gomez R, Otero M, Lago F, Gallego R, Dieguez C, Gomez-Reino JJ, Gualillo O: A new player in cartilage homeostasis: adiponectin induces nitric oxide synthase type II and pro-inflammatory cytokines in chondrocytes. Osteoarthritis Cartilage 2008;16:1101-1109.

2 Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F: Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum 1998;41:778-799.

3 Felson DT: Clinical practice. Osteoarthritis of the knee. N Engl J Med 2006;354:841-848.

4 van den Berg WB, van Riel PL: Uncoupling of inflammation and destruction in rheumatoid arthritis: myth or reality? Arthritis Rheum 2005;52:995-999.

5 Otero M, Goldring MB: Cells of the synovium in rheumatoid arthritis. Chondrocytes. Arthritis Res Ther 2007;9:220.

6 Mitchell NS, Shepard N: Changes in proteoglycan and collagen in cartilage in rheumatoid arthritis. J Bone Joint Surg Am 1978;60:342-348.

7 Mulherin D, Fitzgerald O, Bresnihan B: Clinical improvement and radiological deterioration in rheumatoid arthritis: evidence that the pathogenesis of synovial inflammation and articular erosion may differ. Br J Rheumatol 1996;35:1263-1268.

8 Underwood J.C.E: General and Systematic Pathology. Churchill Livingstone, 2000.

9 Pollard TD, Earnshaw WC: Cell Biology. Saunders, 2004.

10 Charo IF, Ransohoff RM: The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 2006;354:610-621.

11 Baynes JW, Dominiczak MH: Medical Biochemistry. Elsevier mosby, 2005.

12 Serhan CN, Savill J: Resolution of inflammation: the beginning programs the end. Nat Immunol 2005;6:1191-1197.

13 Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, Hendrick AG, Carlton MB, Greaves DR: Synthetic chemerin-derived peptides suppress inflammation through ChemR23. J Exp Med 2008;205:767-775.

14 Cytokines and Joint Injury. Birkhauser, 2004.

15 Knedla A, Neumann E, Muller-Ladner U: Developments in the synovial biology field 2006. Arthritis Res Ther 2007;9:209.

16 Goldring MB, Goldring SR: Osteoarthritis. J Cell Physiol 2007;213:626-634.

17 Tetlow LC, Woolley DE: Comparative immunolocalization studies of collagenase 1 and collagenase 3 production in the rheumatoid lesion, and by human chondrocytes and synoviocytes in vitro. Br J Rheumatol 1998;37:64-70.

18 Barksby HE, Hui W, Wappler I, Peters HH, Milner JM, Richards CD, Cawston TE, Rowan AD: Interleukin-1 in combination with oncostatin M up-regulates multiple genes in chondrocytes: implications for cartilage destruction and repair. Arthritis Rheum 2006;54:540-550.

19 Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M, Mantovani A, Sozzani S, Vassart G, Parmentier M, Communi D: Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. J Exp Med 2003;198:977-985.

20 Caux C, Vanbervliet B, Massacrier C, Ait-Yahia S, Vaure C, Chemin K, Dieu-Nosjean And MC, Vicari A: Regulation of dendritic cell recruitment by chemokines. Transplantation 2002;73:S7-11.

21 Godessart N: Chemokine receptors: attractive targets for drug discovery. Ann N Y Acad Sci 2005;1051:647-657.

22 Iwamoto T, Okamoto H, Toyama Y, Momohara S: Molecular aspects of rheumatoid arthritis: chemokines in the joints of patients. FEBS J 2008;275:4448-4455.

23 Wittamer V, Bondue B, Guillabert A, Vassart G, Parmentier M, Communi D: Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. J Immunol 2005;175:487-493.

24 Szekanecz Z, Kim J, Koch AE: Chemokines and chemokine receptors in rheumatoid arthritis. Semin Immunol 2003;15:15-21.

25 Iwamoto T, Okamoto H, Iikuni N, Takeuchi M, Toyama Y, Tomatsu T, Kamatani N, Momohara S: Monocyte chemoattractant protein-4 (MCP-4)/CCL13 is highly expressed in cartilage from patients with rheumatoid arthritis. Rheumatology (Oxford) 2006;45:421-424.

26 Momohara S, Okamoto H, Iwamoto T, Mizumura T, Ikari K, Kawaguchi Y, Takeuchi M, Kamatani N, Tomatsu T: High CCL18/PARC expression in articular cartilage and synovial tissue of patients with rheumatoid arthritis. J Rheumatol 2007;34:266-271.

27 Lotz M, Terkeltaub R, Villiger PM: Cartilage and joint inflammation. Regulation of IL-8 expression by human articular chondrocytes. J Immunol 1992;148:466-473.

28 Meder W, Wendland M, Busmann A, Kutzleb C, Spodsberg N, John H, Richter R, Schleuder D, Meyer M, Forssmann WG: Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23. FEBS Lett 2003;555:495-499.

29 Parolini S, Santoro A, Marcenaro E, Luini W, Massardi L, Facchetti F, Communi D, Parmentier M, Majorana A, Sironi M, Tabellini G, Moretta A, Sozzani S: The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. Blood 2007;109:3625-3632.

30 Zabel BA, Zuniga L, Ohyama T, Allen SJ, Cichy J, Handel TM, Butcher EC: Chemoattractants, extracellular proteases, and the integrated host defense response. Exp Hematol 2006;34:1021-1032.

31 Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, Butcher EC: Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. J Biol Chem 2005;280:34661-34666.

32 Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, Walder K, Segal D: Chemerin is a novel adipokine associated with obesity and metabolic syndrome. Endocrinology 2007;148:4687-4694.

33 Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, Muruganandan S, Sinal CJ: Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. J Biol Chem 2007;282:28175-28188.

34 Roh SG, Song SH, Choi KC, Katoh K, Wittamer V, Parmentier M, Sasaki S: Chemerin--a new adipokine that modulates adipogenesis via its own receptor. Biochem Biophys Res Commun 2007;362:1013-1018.

35 J.Koolman, K.H.Roehm: Color Atlas of Biochemistry. Thieme Stuttgart, New York, 2003.

36 Zabel BA, Nakae S, Zuniga L, Kim JY, Ohyama T, Alt C, Pan J, Suto H, Soler D, Allen SJ, Handel TM, Song CH, Galli SJ, Butcher EC: Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. J Exp Med 2008;205:2207-2220.

37 Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L: Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889-895.

38 Mognetti B, Moussa M, Croitoru J, Menu E, Dormont D, Roques P, Chaouat G: HIV-1 co-receptor expression on trophoblastic cells from early placentas and permissivity to infection by several HIV-1 primary isolates. Clin Exp Immunol 2000;119:486-492.

39 Figenschau Y, Knutsen G, Shahazeydi S, Johansen O, Sveinbjornsson B: Human articular chondrocytes express functional leptin receptors. Biochem Biophys Res Commun 2001;287:190-197.

40 Ehrlich GE: The rise of osteoarthritis. Bull World Health Organ 2003;81:630.

41 Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, Smolen JS, Weisman M, Emery P, Feldmann M, Harriman GR, Maini RN: Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 2000;343:1594-1602.

42 Ehrlich GE: The subclinical phase of osteoarthritis. J Rheumatol 1987;14 Spec No:88-90.

43 Ehrlich GE: Osteoarthritis beginning with inflammation. Definitions and correlations. 1975. Bull World Health Organ 2003;81:691-693.

44 Barksby HE, Milner JM, Patterson AM, Peake NJ, Hui W, Robson T, Lakey R, Middleton J, Cawston TE, Richards CD, Rowan AD: Matrix metalloproteinase 10 promotion of collagenolysis via procollagenase activation: implications for cartilage degradation in arthritis. Arthritis Rheum 2006;54:3244-3253.

45 Pettersen I, Figenschau Y, Olsen E, Bakkelund W, Smedsrod B, Sveinbjornsson B: Tumor necrosis factor-related apoptosis-inducing ligand induces apoptosis in human articular chondrocytes in vitro. Biochem Biophys Res Commun 2002;296:671-676.

46 Jahn B, Burmester GR, Schmid H, Weseloh G, Rohwer P, Kalden JR: Changes in cell surface antigen expression on human articular chondrocytes induced by gamma-interferon. Induction of Ia antigens. Arthritis Rheum 1987;30:64-74.

47 Mullonkal CJ, Toledo-Pereyra LH: Akt in ischemia and reperfusion. J Invest Surg 2007;20:195-203.

48 Saklatvala J: Inflammatory signaling in cartilage: MAPK and NF-kappaB pathways in chondrocytes and the use of inhibitors for research into pathogenesis and therapy of osteoarthritis. Curr Drug Targets 2007;8:305-313.

49 Klimiuk PA, Sierakowski S: [Cytokines in rheumatoid arthritis. I. Proinflammatory cytokines]. Pol Merkur Lekarski 2001;11:510-513.

50 Schroder JM: The neutrophil-activating peptide 1/interleukin 8, a novel neutrophil chemotactic cytokine. Arch Immunol Ther Exp (Warsz) 1992;40:23-31.

51 Guerne PA, Carson DA, Lotz M: IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. J Immunol 1990;144:499-505.

52 Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, Kishimoto T: Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. EMBO J 1987;6:2939-2945.

53 Lotz M, Jirik F, Kabouridis P, Tsoukas C, Hirano T, Kishimoto T, Carson DA: B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. J Exp Med 1988;167:1253-1258.

54 Jasin HE, Taurog JD: Mechanisms of disruption of the articular cartilage surface in inflammation. Neutrophil elastase increases availability of collagen type II epitopes for binding with antibody on the surface of articular cartilage. J Clin Invest 1991;87:1531-1536.

55 van den Berg WB: Animal models of arthritis. What have we learned? J Rheumatol Suppl 2005;72:7-9.

56 Takaishi H, Kimura T, Dalal S, Okada Y, D'Armiento J: Joint diseases and matrix metalloproteinases: a role for MMP-13. Curr Pharm Biotechnol 2008;9:47-54.

57 Imai K, Ohta S, Matsumoto T, Fujimoto N, Sato H, Seiki M, Okada Y: Expression of membrane-type 1 matrix metalloproteinase and activation of progelatinase A in human osteoarthritic cartilage. Am J Pathol 1997;151:245-256.

58 Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE: Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 1996;97:761-768.

59 Mohtai M, Smith RL, Schurman DJ, Tsuji Y, Torti FM, Hutchinson NI, Stetler-Stevenson WG, Goldberg GI: Expression of 92-kD type IV collagenase/gelatinase (gelatinase B) in osteoarthritic cartilage and its induction in normal human articular cartilage by interleukin 1. J Clin Invest 1993;92:179-185.

60 Okada Y, Shinmei M, Tanaka O, Naka K, Kimura A, Nakanishi I, Bayliss MT, Iwata K, Nagase H: Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. Lab Invest 1992;66:680-690.

61 Shlopov BV, Lie WR, Mainardi CL, Cole AA, Chubinskaya S, Hasty KA: Osteoarthritic lesions: involvement of three different collagenases. Arthritis Rheum 1997;40:2065-2074.

62 Fosang AJ, Last K, Maciewicz RA: Aggrecan is degraded by matrix metalloproteinases in human arthritis. Evidence that matrix metalloproteinase and aggrecanase activities can be independent. J Clin Invest 1996;98:2292-2299.

63 Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G, Nagase H: Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur J Biochem 1990;194:721-730.

64 Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K, Hayakawa T: Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. J Biol Chem 1992;267:21712-21719.

65 Fraser A, Fearon U, Billinghurst RC, Ionescu M, Reece R, Barwick T, Emery P, Poole AR, Veale DJ: Turnover of type II collagen and aggrecan in cartilage matrix at the onset of inflammatory arthritis in humans: relationship to mediators of systemic and local inflammation. Arthritis Rheum 2003;48:3085-3095.

66 Larsen C, Zachariae C, Mukaida N, Anderson A, Yamada M, Oppenheim J, Matsushima K: Proinflammatory cytokines interleukin 1 and tumor necrosis factor induce cytokines that are chemotactic for neutrophils, T cells and monocytes. Prog Clin Biol Res 1990;349:419-431.

67 Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, Baggiolini M, Clark-Lewis I: The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3. J Biol Chem 2001;276:2986-2991.

68 Petkovic V, Moghini C, Paoletti S, Uguccioni M, Gerber B: Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5. A human chemokine with a regulatory role. J Biol Chem 2004;279:23357-23363.

69 Bhalay G, Dunstan A: Chemokine Receptors and Drug Discovery--SMR meeting. 11 March 2004, Horsham, UK. IDrugs 2004;7:441-443.

70 Gladue RP, Zwillich SH, Clucas AT, Brown MF: CCR1 antagonists for the treatment of autoimmune diseases. Curr Opin Investig Drugs 2004;5:499-504.

71 Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH, Hong S, Serhan CN: Molecular circuits of resolution: formation and actions of resolvins and protectins. J Immunol 2005;174:4345-4355.

72 Serhan CN, Savill J: Resolution of inflammation: the beginning programs the end. Nat Immunol 2005;6:1191-1197.

73 Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K: Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med 2000;192:1197-1204.

74 Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac RL: Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. J Exp Med 2002;196:1025-1037.

75 Hasturk H, Kantarci A, Ohira T, Arita M, Ebrahimi N, Chiang N, Petasis NA, Levy BD, Serhan CN, Van Dyke TE: RvE1 protects from local inflammation and osteoclast- mediated bone destruction in periodontitis. FASEB J 2006;20:401-403.

76 Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA, Serhan CN: Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J Exp Med 2005;201:713-722.

77 Arita M, Yoshida M, Hong S, Tjonahen E, Glickman JN, Petasis NA, Blumberg RS, Serhan CN: Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Proc Natl Acad Sci U S A 2005;102:7671-7676.

Appendix

A1. PCR Protocol

Equipment:

5 Prime MasterMix (Cat.no. PRME2200100, 5 prime, VWR international, Oslo, Norway)

Perkin-Elmer GeneAmpPCR system 2400 (Perkin-Elmer, Cambridge, UK)

Remember to preheat the PCR block

- Make the working solution of all the primers to be 1000 nM from the stock:
  Stock primers for ChemR23: 100 uM
  Stock primers for APRT: 100 uM
- Working solution 1000 nmol/L:
  100 000 nmol/L x X = 1000 nmol/L x 500 ul X = 5 ul
  Solve 5 ul of each primer in 495 ul distilled water
- Thaw the mastermix and mix well
- Prepare PCR tubes
- Mix the different templates/primers in sterile 0,2 ml tubes and adjust the volume with water:

Component	**Reaction volume 50 ul**	**End concentration**
Master mix (Taq, dNTP, Mgcl, buffer)	**20ul**	
Template/primer mix	**30 ul**	
Forward primer	7,5 ul	100-200 nM
Reverse primer	7,5 ul	100-200 nM
Template DNA	3 ul	cDNA
Water	12 ul	

- First add the correct volume of master mix to each PCR tube.
- Then add the template/pimer mix to each tube. Mix without making bubbles and place the tubes on ice.
- Run the PCR reaction; Denaturing temp: 94 ºC. Annealing temp 55 ºC at 30 cycles.

- After the run, the PCR may be visualized by gel electrophoresis, ore stored in the freezer for later analyzes.

Sample layout:
Sample 1: Mastermix + APRTF + APRTR + cDNA1 + H2O
Sample 2: Mastermix + APRTF + APRTR + cDNA2 + H2O
Sample 3: Mastermix + APRTF + APRTR + H2O
Sample 4: Mastermix + ChemR23F + ChemR23R + cDNA1 + H2O
Sample 5: Mastermix + ChemR23F + ChemR23R + cDNA2 + H2O
Sample 6: Mastermix + ChemR23F + ChemR23R + H2O
Sample 7: Mastermix + APRTF + APRTR + Genomic DNA + H2O

A1.1 PCR-product visualization
Equipment:
Novex TBE gel 6% (Invitrogen cat no. EC6265BOX)
Xcell surelock mini-cell (Invitrogen cat no. EI0001)
Powerease 500 power supply (Invitrogen cat no. EI8700)
TBE running buffer 5X (Invitrogen cat no. LC6675)
High density TBE loading buffer 5X (Invitrogen cat no. LC6678)
PCR marker (Novagen cat no. 69278-3)
SybrSafe (Cat. No S33102, Invitrogen, Norway
G-BOX (Syngene, Cambridge, UK)

- Make the TBE running buffer 1000 ml: 200 ml 5xTBE + 800 ml deionised wather
- Unwrap and prepare a gel: Take out one gel and rinse with water. Remove the tape from the back of the gel. Remove the well comb and wash the wells three times with running buffer. Place the gel in the upper buffer chamber.
- Install the electrophoresis chamber: fill the upper chamber with 200 ml running buffer and the lower chamber with 600 ml running buffer.
- Add loading buffer to the PCR samples to a total volume of 25ul:

15 ul PCR product

5 ul sample loading buffer

5 ul deionized water

25ul Total volume

Well: 1: 5 ul Marker
2: 25 ul sample
3: 25 ul sample 2
4: 25 ul sample 3
5: 25 ul sample 4
6: 25 ul sample 5
7: 25 ul sample 6
8: 25 ul sample 7
9: 5 ul Marker

- Place the lid to the chamber and connect the power supply.
- Choose a pre-maid program for TBE gels on the “power ease 500” and press start.
- After the run; incubate the gel in SybrSafe solution for 30 minutes.
- Incubate the gel in water for 30 minutes before visualizing the gel bands with the gel documentation system.

A2. Immunecytochemistry protocol

Equipment:

Lab-TekII chamber slides (Cat. No. 154534 Nunc, VWR international, Norway)

Fibronectin (Cat. No. F4759, SigmaAldrich)

Fixation buffer (PBS with 0,2 M sucrose + 4% paraformaldehyd)

ChemR23 antibody (Cat. No.ab13172, Abcam, Cambridge, UK)

Secondary antibodies (Alexa fluor 488 goat anti-rabbit IgG, Cat No. A-11008 and Alexa fluor 568 goat anti-rabbit IgG, Cat. No. A-11011, Invitrogen, Oslo, Norway)

Dapi-Fluoromount-G (Cat. No. 0100-20, SouthernBiotech)

Day 1:

- Coat one slide with fibronectin by covering the slide with fibronectin solution, remove all excess liquid and let the slide dry up.
- Wash the slide with 200 ul PBS in each well.
- Make 2 ml growth medium with 5 % FCS: 2000 ul medium + 100 ul FCS
- Add 400 ul of the growth medium to each well of the slide. Add 20 ul of the cell suspensjon to each well. Check in the microscope to see if the cell number is preferable.
- Incubate the slides in a cell incubator for 24 hours.

Day 2:

- Wash the cells carefully two times with PBS
- Fixate the cells by adding 200 ul cold fixation buffer and incubate the slide for 1 hour in 4 ºC.
- Wash the slide two times
- Add 200 ul PBS with 1% BSA to each well and incubate for 1 hour.
- Wash the slide two times
- Add 200 ul primary antibody (1:250) to each well exept for the two control wells. Incubate the slide over night at4 ºC. Add 200 ul PBS to the control well

Slide layout:

Primary antibody: Rabbit anti-human chemR23 1:250

Secondary antibody: Goat anti- rabbit IgG alexa fluor 488 1:250

Goat anti-rabbit IgG alexa fluor 568 1:250

Control 488	Control 488
Test 488	Test 488
Test 568	Test 568
Control 568	Control 598

Day 3:

- Wash the slide two times
- Add 200 ul secondary antibody (1:200) and incubate the slide in 1 hour in room temperature.
- Wash three times
- Remove all the wells and Add Dapi-Fluromount-G before covering the slide with a cover glass
- Let the slide set for 30 minutes
- Microscope the slides

A3. Western blotting protocol

Equipment:

Electrophoresis: Xcell surelock Mini-cell (Cat. No. EI0001 Invitrogen)

Blotting module: XCell II Blot module (EI9051 Invitrogen)

NuPAGE MESS DS buffer kit (Cat. No. NP0060, Invitrogen)

Halt Phosphatase inhibitor coctail (Cat. No.78420, Thermo)

Protease inhibitor (Cat.No. 04693124001, Roche, Indianapolis, US)

Hepatocytometer

MEK 1/2 inhibitor (Cat. No. U0126, Cell Signaling)

Recombinant chemerin (Cat. No. 2324-CM, R&D)

Trypsin (Cat. No T-3924, Sigma)

Day 1

- Remove the medium from cultivated chondrocytes
- Add 5 ml PBS and wash the cells
- Remove the PBS
- Repeat step 2 and 3
- Add 1 ml trypsin, and watch the cell in the microscope to see if they have loosened.
- Add 5 ml medium with 10% FCS and transfer the suspension to a 15 ml tube for centrifugation.
- Centrifuge for 5 min in 1500 RPMI, remove the supernatant and add 5 ml of new medium
- Count the cells to determine the cell number:

  Burkers counting chambre: 1E square correspond to 1/250 ul = 0,004 ul

  The chamber consists of 144 E squares and this correspond to 0,576 ul

  Count 10 E squares:

  Cell count per square: N/10

  Cell count per ul: $\frac{(N/10)}{0{,}004\ ul}$ = celles/ul

  Total cell count: celles/ul x volume of cell suspension in ul

- Transfer the amount of ul that accounts for 0.5 x 10^6 cells to each well with total of 6 wells. Add fresh medium with 10 % FCS to a total wolume of 3 ml. Incubate the cells over night.

Day 2

- Remove the medium from each well and wash carefully with PBS. Add 3 ml fresh medium without FCS. Incubate the cells overnight.

Day 3

- Remove the medium in one well and add 10 uM MEK 1/2 inhibitor in 2 ml medium one hour prior to the stimulation
- Remove the medium in all the wells except the inhibitor well, and add the chemerin and medium to its respective wells (see cell stimulation protocol further down)

- Remove the medium and stimuli at the different time intervals and wash once with PBS.
- Remove all liquid before the sample buffer is added
- Add the sample buffer and reducing agent directly into the wells:

Add 150 ul 1x sample buffer with 100mM DTT to each well:

240 ul Sample buffer 4x + 720 ul deionised water

Add DDT to a 1X concentration = 1 ul per 10 ul

960 ul / 10 = 96 x 1 = 96 ul DDT

96 ul DDT 10x + 864 ul sample buffer 1X

Add 10 ul phosphatase inhibitor cocktail and 20 ul protease inhibitor cocktail to the sample buffer before adding it to the wells.

- Scrape the wells and collect the fluid into 1.5 ml tubes on ice
- Sonicate the tubes in 10-15 seconds
- Centrifuge the tubes for 10 min at 14 000 rpm, collect the supernatant for use.

A3.1 Cell stimulation for western blot

MEK1/2 Inhibitor:

Stock: 10 mM

Final concentration in on well with a total volume of 2 ml:

2 ml x 10 umol/l = X x 10 000 umol/l

X = 0,002 ml = 2 ul

Chemerin

Stoch: 25 ug

Solve in 1 ml PBS with 0,1% BSA = 25ug/ml = 0,025 mg/ml = 0,025 g/l

Mw = 16 000 g/mol

Stock will be:

$\frac{0{,}025 \text{ g/l}}{16000 \text{ g/mol}} = 0{,}00000156 \text{ M} = 0{,}00156 \text{ mM}$

Make 9 ml of a 10 nM chemerin solution:

9 ml x 0,00001 mmol/l = X x 0,00156 mmol/l

X = 0,0576

X = 57,6 ul

Add 58 ul Chemerin stock to 8,95 ml medium without serum

Plate layout:

Well 1: Control 2000 ul medium + 12,8 ul PBS/0,1 % BSA	Well 2: MEK1/2 3 min 2000 ul medium/Inhibitor + 12,8 ul stock chemerin	Well 3: 1 min 2000 ul medium/ 10 nM chemerin
Well 4: 2,5 min 2000 ul medium/ 10 nM chemerin	Well 5: 5 min 2000 ul medium/ 10 nM chemerin	Well 6: 10 min 2000 ul medium/ 10 nM chemerin

A3.2 Electrophoresis

Equipment:

NuPAGE Novex 4-12 % Bis-Tris gels 15 brønner (Cat. No. NP0323, Invitrogen)

Prestained protein marker (Cat. No. 7720 CellSignaling)

Biotinylated protein ladder (Cat. No. NP 7727 cellsignaling)

Running buffer (Cat. No. NP 0002 Invitrogen)

Antioxidant (Cat. No. NP0005 Invitrogen)

- Make 1 litre of 1x Running buffer: 50 ml 20x running buffer + 950 ml deionised water
- Open one gel and rinse with water
- Remove the comb from the wells and wash the wells three times with running buffer.
- Remove the gel tape
- Set up the gel in the electrophoresis chamber.
- Fill the inner upper chamber with 200ml running buffer and add 500ul antioxidant
- Fill the lower chamber with 600 ml running buffer
- Boil the samples and ladders at 95-100 ºC for 3 min
- Add samples and ladders to their respective wells
- Run the gel

Gel layout:

Well	Sample
1	Prestained ladder 15 ul
2	Biotinylert ladder 10 ul
3	Test 0 min 15 ul
4	MEK 1/2 Inhibitor
5	Test 1 min 15 ul
6	Test 2,5 min 15 ul
7	Test 5 min 15 ul
8	Biotinylert ladder 10 ul

A3.3 Blotting procedure

Equipment:

PVDF membrane 0,45uM (Cat. No. LC2005 Invitrogen)

NuPAGE transfer buffer 20x (Cat.No. NP0006 Invitrogen)

Methanol

- Make 1x transfer buffer: 50 ml 20x transfer b + 100 ml methanol + 849 ml deionised water + 1 ml antioxidant
- Prepare the PVDF- membrane:
- wet the membrane in methanol
- rinse in water
- incubate the membrane in transfer buffer for minimum 5 min
- Open the gel-cassette and remove the wells
- Place a pre-wet filter paper on the gel and flip the gel into your hand, resting on the filter-paper.
- Remove the lower part of the gel so it loosens from the cassette.
- Wet the gel with transfer buffer and place the PVDF membrane on the gel followed by a filter-paper. Remove air bubbles
- Place to pre-wet blotting pads into the cathode (-) in the blotting module.

- Place the gel and the membrane with the filter-papers over the pads, with the gel resting closest to the cathode.
- Place two pre-wet blotting pads over the gel and membrane.
- Place the anode (+) on top
- Place the hold module in the electrophoresis chamber
- Fill the module chamber with transfer buffer
- Fill the outer chamber with 650 ml of water
- 16. Run the electro-blotting

A3.4 Detection

Equipment:

Phototope-HRP WB detection system (Cat. No. 7071 CellSignaling)

Phospho-Erk1/2 Pathway sampler kit (Cat. No. 9911 Cell Signaling)

TBS buffer: Tris Base, NaCL

Blocking buffer: Tween, fat fri dry-milk,

Wash buffer (TBS + tween)

Make the reagents:

TBS buffer 10X 250 ml:

6,05 g Tris base

20 g NaCL

250 ml milliQ

Adjust pH to 7,6 buy using HCl

TBS buffer 1X 250 ml:

25 ml 10X TBS buffer

225 ml MilliQ water

Blocking buffer 50 ml:

5 ml 1X TBS

45 ml MilliQ water

2,5 g dry milk (non fat)

0,05 ml (50 ul) Tween-20(100 %)

Wash buffer 100 ml:

0,1 ml (100 ul) Tween-20 0,1 % = (100 x 0,1)/100 = 0,1 ml

100 ml TBE 1X

Primary Antibody dilution buffer 20 ml:

2 ml 10X TBS

18 ml MilliQ water

1.0 g BSA

20 ul Tween-20 (100 %)

- After blotting, wash the membrane in 5 ml 1x TBS in 5 minutes
- Incubate the membrane in 10 ml blocking buffer for one hour
- Wash 3x5 min in 5 ml wash buffer
- Incubate the membrane with 10 ml primary antibody over night at 4 ºC

Dilution phospho-p44/42 MAPK antibody, 42,44 kDa:	1:2000 in 10 ml = 5 ul Ab
Dilution phospho-Akt antibody, 60 kDa	1:1000 in 10 ml = 10 ul Ab
Add 15 ul total of the two antibodies in 10 ml dilution buffer	

Day 4

- Remove he primary antibodies and wash 3x5 min in wash buffer
- Add the secondary antibodies in 10 ml blocking buffer and incubate for one hour in room temperature.

Anti-Rabbit IgG HRP-linked antibody:	1:2000 in 10 ml = 5 ul Ab
Anti-Biotin HRP linked:	1:1000 in 10 ml = 10 ul i Ab
Add 15 ul total of the two antibodies in 10 ml dilution buffer	

- Wash 3x 5 min in 5 ml wash buffer
- Add 5 ml LumiGLO (250ul 20X LumiGlo + 250ul 20x Peroxide + 4,5 ml milliQ water)

- Mix for 1 minute
- Rap the membrane in plastic and develop the membrane

A4. Cytokine measurement protocol

A4.1 Cell stimulation

Day 1

- Remove the medium from cultivated chondrocytes
- Add 5 ml PBS and wash the cells
- Remove the PBS
- Repeat step 2 and 3
- Add 1 ml trypsin, and watch the cell in the microscope to see if they have loosened.
- Add 5 ml medium with 10% FCS and transfer the suspension to a 15 ml tube for centrifugation.
- Centrifuge for 5 min in 1500 RPM, remove the supernatant and add 5 ml of new medium
- Count the cells to determine the cell number:

  Burkers counting chamber: 1E square correspond to 1/250 ul = 0,004 ul

  The chamber consists of 144 E squares and this correspond to 0,576 ul

  Count 10 E squares:

  Cell count per square: N/10

  Cell count per ul: $\frac{(N/10)}{0{,}004\ ul}$ = celles/ul

  Total cell count: celles/ul x volume of cell suspension in ul

- Transfer the amount of ul that accounts for $0.5x10^6$ cells to each well with total of 3 wells. Add fresh medium with 10 % FCS to a total volume of 3 ml. Incubate the cells over night.

Day 2

- Remove the medium from each well and wash carefully with PBS. Add 3 ml fresh medium without FCS. Incubate the cells overnight.

Day 3

- Remove the medium from all wells and wash once with PBS
- Add Chemerin and medium to its respective wells (se plate layout further down)
- Incubate the plate for 24 hours

Day 4

- Harvest the cellsupernatants and centrifuge the tubes for 5 min at 10 000 RPM to remove cell fragments
- Aliqout the supernatant into five cryotubes and freeze the tubes in minus 70 ºC until analysis.

Plate layout:

Well 1: Control Cells + 1000 ul medium with PBS/0,1 % BSA	Well 2: 10 nM Cells + 1000 ul medium/10 nM chemerin	Well 3: 100 nM Cells + 1000 ul medium/100 mM chemerin

A4.2 Quantitatively determination of cytokines

Equipment:

Bio-plex cytokine assay (Cat No. X50053UVBS, Bio-Rad, Hercules, CA)

Bio-plex 200 system (Bio-Rad, Hercules, CA)

- Solve the standard: The standard has to be solved in the same matrix as the samples. Add 500 µl cell growth medium, vortex gently for a few seconds and leave the standard on ice for 30 min.
- Prepare the samples:

  The samples should be run at a 1:4 dilution

  1 : 4: 30µl cell sample + 90µl diluent

- Make the standard curve:

  Standard values for standard 1 is printed in the std package. The standard dilution is 1:4

  Std 1: 128 µl stock + 72 µl medium

  Std 2: 50µl std1 + 150µl medium

  Std 3: 50µl std2 + 150µl medium

  Std 4: 50µl std3 + 150µl medium

  Std 5: 50µl std4 + 150µl medium

  Std 6: 50µl std5 + 150µl medium

  Std 7: 50µl std6 + 150µl medium

  Std 8: 50µl std7 + 150µl medium

  Std 9: 50µl std 8 + 150µl medium

- Pre-wet the 96-well plate:

  Add 100µl assay buffer to each ell
- Remove the buffer by vacuum
- Add 50 ul of the assay beads to each well
- Remove the liquid by vacuum and wash the beads twice in wash buffer
- Add standards and samples in their respective wells.
- Seal the plate with plastic, wrap it in foil and incubate on a plate shaker at 300 RPM for 45 min
- Turn on the Bio-Plex machine and prepare for running the plate.
- Dilute the detection antibody:

Detection Antibody (10x)			
Wells	10x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	300	2,700	3,000
48	150	1,350	1,500
32	100	900	1,000
24	75	675	750

- Wash the plate three times by adding 100 ul wash buffer
- Add 25 ul of detection antibody to each well and incubate the plate in foil on a plate shaker for 30 min
- Dilute the Streptavidin-PE:

Wells	Streptavidin-PE (100x) (uL)	ASSAY Buffer A (uL)	Total Volume (uL)
96	60	5 940	6 000
48	30	2 970	3 000
32	20	1 980	2 000
24	15	1 485	1 500

- Wash the plate three times by adding 100 ul wash buffer
- Add 50 ul of strepdavidin-PE to each well and incubate the plate in foil for 10 minutes on a plate shaker.
- Wash the plate three times by adding 100 ul wash buffer
- Add 125 µl assay buffer to each well
- Shake the plate for 1 minute, then analyze the plate

A5. MMP-panel measurement protocol

A5.1 Cell stimulation

Day 1

- Remove the medium from cultivated chondrocytes
- Add 5 ml PBS and wash the cells
- Remove the PBS
- Repeat step 2 and 3
- Add 1 ml trypsin, and watch the cell in the microscope to see if they have loosened.
- Add 5 ml medium with 10% FCS and transfer the suspension to a 15 ml tube for centrifugation.
- Centrifuge for 5 min in 1500 RPM, remove the supernatant and add 5 ml of new medium
- Count the cells to determine the cell number:

  Burkers counting chamber: 1E square correspond to 1/250 ul = 0,004 ul

  The chamber consists of 144 E squares and this correspond to 0,576 ul

Count 10 E squares:

Cell count per square: N/10

Cell count per ul: $\frac{(N/10)}{0{,}004\ ul}$ = celles/ul

Total cell count: celles/ul x volume of cell suspension in ul

- Transfer the amount of ul that accounts for $0.5x10^6$ cells to each well with total of 3 wells. Add fresh medium with 10 % FCS to a total volume of 3 ml. Incubate the cells over night.

Day 2

- Remove the medium from each well and wash carefully with PBS. Add 3 ml fresh medium with 0.1 % FCS. Incubate the cells overnight.

Day 3

- Remove the medium from all wells and wash once with PBS
- Add Chemerin and medium to its respective wells (se plate layout further down)
- Incubate the plate for 48 hours

Day 5

- Harvest the cellsupernatant from the 48 hours incubation plate, centrifuge the tubes for 5 min at 10 000 RPM to remove cell fragments
- Aliqout the supernatant into five cryotubes and freeze the tubes in minus 70 ºC until analysis.

Plate layout:

Well 1: Control Cells + 1000 ul medium with PBS/0,1 % BSA	Well 3: 10 nM Cells + 1000 ul medium/10 mM chemerin	Well 2: 100 nM Cells + 1000 ul medium/100 nM chemerin

A5.2 Quantitatively determination of MMP

Equipment:

Human Base Kit MMP panel Fluorokine MAP (Cat. No. LMP000, R&D)

8 Different Human MMP Fluorokine MAP (Cat. No. LMP901, LMP902, LMP513, LMP907, LMP908, LMP911, LMP919, LMP511).

- Prepare the wash buffer
- Solve the standard in 0,9 ml of calibrator diluent RD5-37 and let it sit for 15 min on ice
- Mix the bead buffer: add 25 ul of each bead buffer (25 ul x 8) into 2300 ul microparticle diluent
- Prepare the samples:
  The samples should be run at a 1:4 dilution
  1:4: 30µl cell sample + 90µl calibrator diluent RD5-37
- Make the standard curve:
  Standard values for standard 1 are printed in the package. The standard dilution is 1:3
  Std 1: 500 µl stock
  Std 2: 100µl std1 + 200µl Calibrator diluent
  Std 3: 100µl std2 + 200µl Calibrator diluent
  Std 4: 100µl std3 + 200µl Calibrator diluent
  Std 5: 100µl std4 + 200µl Calibrator diluent
  Std 6: 100µl std5 + 200µl Calibrator diluent
  Std 7: 100µl std6 + 200µl Calibrator diluent

- Pre-wet the 96-well plate:
  Add 100µl assay buffer to each ell
- Remove the buffer by vacuum
- Add 50 ul of the assay beads to each well
- Add standards and samples in their respective wells.
- Seal the plate with plastic, wrap it in foil and incubate on a plate shaker at 500 RPM for 2 hours
- Turn on the Bio-Plex machine and prepare for running the plate.

- Dilute the detection antibody: Add 50 ul of each antibody into the bottle with ready antibody diluent
- Wash the plate three times by adding 100 ul wash buffer
- Add 50 ul of detection antibody to each well and incubate the plate in foil on a plate shaker for 60 min
- Dilute the Streptavidin-PE: 27,5 ul streptavidin + 2750 ul wash buffer
- Wash the plate three times by adding 100 ul wash buffer
- Add 50 ul of strepdavidin-PE to each well and incubate the plate in foil for 30 minutes on a plate shaker.
- Wash the plate three times by adding 100 ul wash buffer
- Add 100 µl assay buffer to each well
- Shake the plate for 1 minute, then analyze the plate