Aus dem Lehrstuhl für Innere Medizin I
Prof. Dr. Martina Müller-Schilling
der Fakultät für Medizin

der Universität Regensburg

The Endocannabinoid System Exerts Anti-Inflammatory and Pro-Apoptotic Effects on Synovial Fibroblasts in Rheumatoid Arthritis

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Martin Apitz

2015

Aus dem Lehrstuhl für Innere Medizin I
Prof. Dr. Martina Müller-Schilling
der Fakultät für Medizin

der Universität Regensburg

The Endocannabinoid System Exerts Anti-Inflammatory and Pro-Apoptotic Effects on Synovial Fibroblasts in Rheumatoid Arthritis

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Martin Apitz

2015

Dekan:	Prof. Dr. Dr. Torsten E. Reichert
1. Berichterstatter:	Prof. Dr. Rainer H. Straub
2. Berichterstatter:	Prof. Dr. Dr. h.c. Joachim Grifka
Tag der mündlichen Prüfung:	10.11.2015

Contents

Deutsche Zusammenfassung

Die Rheumatoide Arthritis ist eine häufige Autoimmunerkrankung, die 0,5-1 % der erwachsenen Bevölkerung betrifft und als chronische Gelenkentzündung zu fortschreitender Gelenkzerstörung und systemischen Symptomen wie z.B. das metabolisches Syndrom und Atherosklerose führt. In den letzten Jahrzehnten wurde das pathogenetische Verständnis der Rheumatoiden Arthritis auf Nicht-Immunzellen erweitert. So besetzen Fibroblasten der artikulären Synovia eine herausragende Stellung in der Entwicklung der Gelenkentzündung. Im Zusammenspiel mit Immunzellen und Osteoklasten entwickelt sich im chronisch entzündeten Gelenk ein Gewebetumor aus tumorös veränderten Fibroblasten, der Pannus. Diese veränderten Fibroblasten sind verantwortlich für die Synthese von Matrix-Metalloproteasen MMP, die Kapsel- und Knorpelmatrix degradieren. Die Fibroblasten halten die lokale Arthritis aufrecht und koordinieren die Zerstörung angrenzenden Knochens durch die Osteoklasten. Die Unterbindung immunologischer Kommunikation durch sogenannte Biologika wie z.B. TNFα-Hemmer, hat das therapeutische Ergebnis deutlich verbessert, die therapeutischen Kosten jedoch auch deutlich erhöht und schwerwiegende Nebenwirkungen der Immunsuppression eingeführt. Deshalb besteht weiterhin die Notwendigkeit, alternative und adjuvante Therapien zu entwickeln, die nicht nur auf das Immunsystem sondern auch auf nicht-immunologische Zellen wie die Fibroblasten abzielen. Einer der wichtigsten therapeutischen Ergebnisse muss es sein, dass diese Fibroblasten weniger aggressiv wachsen und weniger gewebezerstörende MMP bilden. Intrazellulär wird die Synthese von MMP hauptsäch-

lich durch mitogen-aktivierte Proteinkinasen MAPK gesteuert. Dieses System besteht aus Proteinkinasen, die sich nach extra- oder intrazellulärem Signal konsekutiv und exponentiell aktivieren. Grundsätzlich lassen sich hierbei drei Kinase-Achsen unterscheiden: die Extracellular-Signal Regulated Kinase ERK, die hauptsächliche für Zellwachstum und Zelldifferenzierung verantwortlich ist, sowie die p38 MAPK und c-Jun N-Terminal Kinase JNK, die die zelluläre Antwort auf Stress koordinieren. Assoziiert ist das cAMP-responsive Binding Protein CREB, das ebenso durch zellulären Stress aktiviert wird.
Somit kann ein therapeutischer Ansatz die Reduktion der Aktivität des MAPK-System in den synovialen Fibroblasten sein, um damit die Produktion von MMP zu reduzieren und die Gelenkszerstörung zu verlangsamen.
Das endogene Cannabinoidsystem ECS wurde im Zuge der Cannabis-Forschung entdeckt und stellt ein komplexes Signalsystem aus Lipiden dar. Es besteht neben den Cannabinoid-Rezeptoren CB_1 und CB_2 aus den endogen produzierten Liganden Anandamid und 2-Arachidonylglycerol. Dieses System moduliert sowohl zentralnervös die Nahrungsaufnahme als auch peripher das Immunsystem. Es konnte gezeigt werden, dass synthetisch hergestellte Cannabinoide in Immunzellen und Nicht-Immunzellen einiger chronischen Entzündungen, wie der Rheumatoide Arthritis, suppressiv auf die Aktivität der MAPK und Produktion von MMP wirken. Hingegen wurde die Rolle der endogen produzierten Liganden in der Rheumatoiden Arthritis bislang nur unzureichend erforscht.
Diese Arbeit verwendete humane Fibroblasten aus Patienten mit Rheumatoider Arthritis oder degenerativer Arthrose. Im normoxischen und hypoxischen Umfeld wurden diese Zellen mit selektiven CB_1- und CB_2-Agonisten sowie dem endogen produzierten Anandamid vorbehandelt und zuletzt mit den pro-inflammatorischen Zytokinen TNFα, Interleukin-1β oder dem Endotoxin Lipopolysaccharid stimuliert. Die Aktivität der intrazellulären MAPK ERK, p38, c-Jun und der cAMP-responsive Binding Protein CREB wurde mittels Enzyme-linked Im-

munosorbent Assay ELISA und Proteome Profiler™ gemessen. Des weiteren wurde der Anteil apoptotischer und nekrotischer Zellen mittels Durchflusszytometrie und Messung der Laktatdehydrogenase sowie die Synthese einiger immunmodulatorischer Zytokine mit dem Proteome Profiler™ bestimmt.
Diese Arbeit zeigte, dass der CB_1-Agonist die Aktivität der MAPK nach Zytokin-Stimulation signifikant erhöhte, der CB_2-Agonist jedoch die Aktivität von MAPK und CREB signifikant erniedrigte. Das endogen produzierte Anandamid wirkte in hohen Konzentration (1 µM) wie der CB_1-Agonist pro-inflammatorisch, in niedrigen Konzentration (0.1 nM) jedoch wie der CB_2-Agonist anti-inflammatorisch. Anandamid konnte ebenso die Produktion von pro-inflammatorischen Zytokinen nach Stimulation mit TNFα reduzieren. Des weiteren induzierten sowohl der CB_1-Agonist als auch Anandamid Apoptose und Nekrose in den Fibroblasten.
Erstmals konnte in dieser Arbeit gezeigt werden, dass das endogen produzierte Cannabinoid Anandamid konzentrationsabhängig entweder pro- oder anti-inflammatorisch auf synoviale Fibroblasten wirkt. Mögliche adjuvante Therapien mit Cannabinoiden gegen die Rheumatoide Arthritis müssen somit die lokale Konzentration von endogenen Cannabinoiden und daher die Aktivität des ECS in der Arthritis berücksichtigen. Eine klare Aussage ist in Anbetracht der Komplexität des ECS sowie fehlender in-vivo-Experimente noch nicht möglich. Jedoch stellt die Modulation des ECS mit seinen vielfältigen Wirkungen nicht nur auf Immunzellen, sondern auch auf Nicht-Immunzellen, wie die Fibroblasten, ein mögliches zukünftiges Therapieziel gegen die Rheumatoide Arthritis dar.

Introduction

2.1 Rheumatoid Arthritis: Think Global, Act Local

2.1.1 General Definition

Rheumatoid arthritis is a chronic, autoimmune-induced subset of diseases of yet-unknown origin, which is characterised by

a systemic inflammation, with antibody production as well as pulmonary or cardiovascular complications and

a local inflammation, in the joint with cartilage and bone destruction of small joints of hand and feet,

(McInnes et al., 2011) which altogether leads foremost to debilitating deformities of hands and feet, alongside a 3.17-fold risk of myocardial infarction (Maradit-Kremers et al., 2005) and stroke events, both with more lethal outcome (Davis et al., 2008). These extra-articular complications are largly responsible for higher mortality ratios ranging from 1.28 to 2.98, compared with the normal population (Gabriel et al., 2009).

2.1.2 Epidemiological and Economic Relevance

Rheumatoid arthritis affects around 0.5-1 % of the adult population in developed countries (Gabriel et al., 2009). Due to the long-term therapy required, the debilitating course of the disease and the high comorbidity, mean healthcare expenditures per patient with RA per year in Europe and Northern America are estimated to be € 4000-6000. In Germany, expenditures for patients with RA range from € 2437–2981

annually per patient of working age, to an estimated € 2121 per year for patients in retirement, as of 2002 (Furneri et al., 2012; Huscher et al., 2014).
Although the new biological therapeutics have overall reduced disease activity and, concomitantly, hospitalisation and work absence, the economic benefit was counterbalanced by 3- to 6-fold increases in treatment cost (Ziegler et al., 2010; ter Wee et al., 2012; Hagel et al., 2013).
Despite these positive trends in the therapeutic outcome, functional debilitation and thus work absence are still severe in rheumatoid arthritis, so that these patients still rank among patients with chronic ischaemic heart disease and multiple sclerosis, with respect to reduction of health utility in quality of life (Huscher et al., 2006; Furneri et al., 2012).
These stastistics demonstrate an ongoing need for new, less expensive combinations of treatments to reduce climbing expenditures and economic burden, to help decreasing disease activity, and to foster quality of life and long-term survival for patients with rheumatoid arthritis.

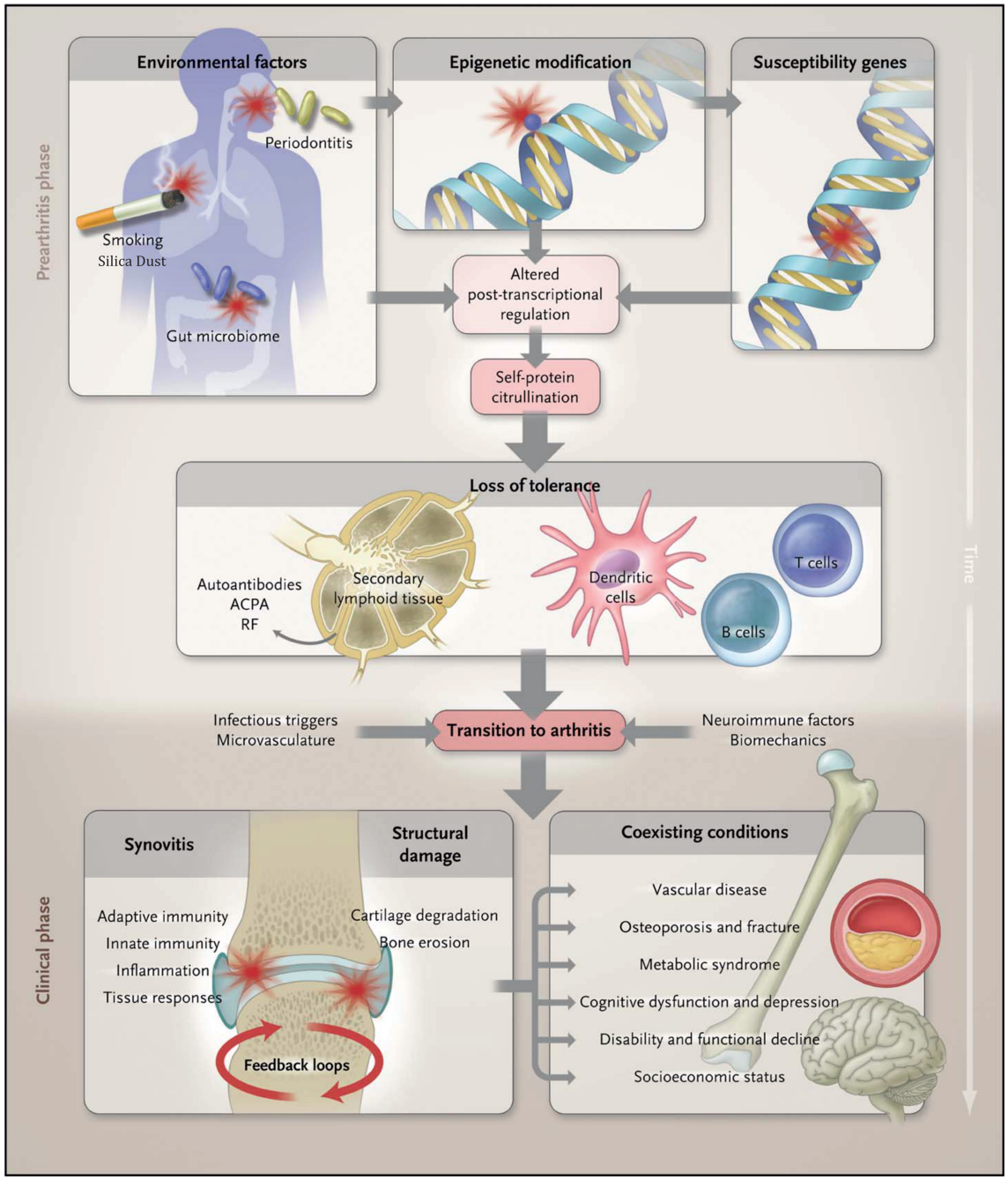

Figure 2.1: The Multistep Pathogenesis of Rheumatoid Arthritis (McInnes et al., 2011). ACPA denotes anti-citrullinated peptide antibodies and RF rheumatoid factor. *Silica Dust* was added to the original picture.

2.2 Pathogenesis of Rheumatoid Arthritis

In recent years, our insight into the pathogenesis and etiology of RA has increased tremendously. With different clinical presentations and distinct genetic and environmental risk factors, RA can now be seen as a group of different diseases that share a common final pathway of inflammation and joint destruction (Scott et al., 2010; van der Helm-van Mil et al., 2008).
Although a comprehensive pathogenetic model has not yet been developed, evidence is now enlarging the picture and including mesenchymal cells like fibroblasts and osteoclasts in the inflammatory process. McInnes and Schett (McInnes et al., 2011) present three crucial phases in the development of RA (Figure 2.1).

Prearthritic Phase: Genetic predisposition, environmental exposure and combined epigenetic alteration lead to autonomous protein citrullination.

Loss of Tolerance Phase: Auto-antibodies are processed and secreted, especially ACPA against citrullinated proteins.

Arthritic Phase: Further biomechanical, microvascular, infectious or neuroendocrinological factors result in the clinical presentation of local and systemic inflammation.

2.2.1 Prearthritic Phase

Genetic Predisposition Twin studies revealed concordance rates of 15-30 % in monozygotic twins against 5 % in dizygotic twins (MacGregor et al., 2000). Currently, the most important filial risk factor are HLA-DRβ chain alleles that have the common protein sequence QKRAA, which is called the *shared epitope* (Gregersen et al., 1987). The biological link to an increased susceptibility is still unknown. However, the shared epitope works as a signal-transducing ligand for calreticulin in leukocytes, which plays a vital role in the elimination of apoptotic cells and can therefore be regarded as crucial for the devel-

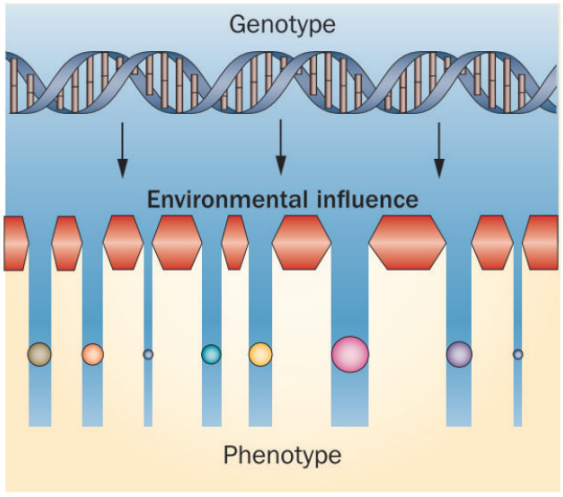

Figure 2.2: The Mechanism of Epigenetics in Rheumatoid Arthritis (Grabiec et al., 2013)

opment of peripheral autoimmunity (Almeida et al., 2010). Further risk factors for rheumatoid arthritis include genetic modifications in the STAT4 signaling pathway (Remmers et al., 2007) and in T-cell activation, e.g. via PTPN22 (Begovich et al., 2004). As the low concordance rates for mono- and dizygotic twins above suggest, the exposure to diverse environmental factors mainly influence the pathogenesis and onset of RA.

Environmental Influence In addition, a host of infectious and non-infectious causes have been marked as risk factors for developing rheumatoid arthritis (see Table 2.1). Particularly, environmental exposure to bronchial stress such as smoking or silica dust increases the risk of developing RA in patients with susceptibility HLA alleles, such as HLA-DR1 and the shared epitope sequence or other HLA-DR4 alleles (Klareskog et al., 2006; Källberg et al., 2011). The bronchial stressors might induce citrullination of mucosal proteins through post-translational processing and modification of e.g. the peptidyl arginine deiminase type IV PADI4, which is required for ACPA development, the most specific auto-antibody involved in RA (McInnes et al., 2011). Also, the new field of epigenetics might provide insight into

this field of gen-environment interactions in the future.

Linking together: Epigenetics The transcription is regulated mainly by the three mechanisms: DNA methylation, histone acetylation and miRNA expression. These so-called epigenetic changes are responsible for the access and binding of transcription factors and therefore create a phenotype without altering the DNA code (Grabiec et al., 2013). They are considered crucial in two ways (Bottini et al., 2013):

Etiologically, they provide a linkage mechanism: they explain how environmental exposure over time and family genetic predispositions create an individual risk for rheumatoid arthritis. The epigenetic machinery functions as a filter, which selects gene expression and therefore the cell phenotype, depending on the environmental circumstances (Figure 2.2).

Pathogenically, they are involved in the transformation of leukocytes and especially synovial fibroblasts into aggressive behaviour, which is described in detail in Section 2.3.

As a consequence, external influences and internal genetic predispositions in the development of RA cannot be clearly differentiated. The personal and local environment can have a long-term impact on the internal genetic susceptibility to RA, therefore modulating its individual risk and variability.

2.2.2 Loss of Tolerance Phase

Citrullination α-Enolase, keratin, fibrinogen, fibronectin, collagen, and vimentin are prone to self-citrullination, against which ACPA antibodies are secreted. Citrullinated α-enolase is highly associated with ACPA-positive RA as well as the risk factors described in Section 2.2.1 (Mahdi et al., 2009). ACPA itself has been shown to be one of the highest predictors of early-onset RA (van der Linden et al., 2009).

Transition to Arthritic Phase It remains unclear why the systemic loss of tolerance, alongside with the prodution of auto-antibodies such

Table 2.1: Environmental Risk Factors for Rheumatoid Arthritis

Associated Factor	Mechanistic Link	References
Infectious Causes		
Various Bacterial and Virus Infection	Molecular Mimikry, such as microbial HSP60 as an epitope to HLA-DR4	(Kamphuis et al., 2005)
Periodontal Infection with Porphyromonas gingivali	Porphyromonas gingivali produces PADI4, therefore promoting citrullination of α-enolase and fibrinogen	(Wegner et al., 2010)
Altered Gastrointestinal Microbiota	Gastrointestinal bacteria might influence the epithelial and mucosal permeability and the local immune cells	(Yeoh et al., 2013)
Non-infectious Causes		
Bronchial stress such as smoking or silica dust	Bronchial stressors such as smoking (21-fold risk for RA) or silica dust (1.5- to 2.5-fold risk alone, 7-fold risk together with smoking) lead to citrullination of mucosal proteins through post-translational processing and modification of e.g. PADI4, which induces the development of ACPA	(Klareskog et al., 2006; Stolt et al., 2010)
Adverse life events and chronic inflammation	Neuroimmunological involvement along the hypothalamic-pituitary-adrenal stress system and sympathetic nerve system, leading to a dysbalance and increase of energy allocation to the immune system	(Capellino et al., 2010; Straub et al., 2010; Stefanski et al., 2013; Straub, 2014)

as ACPA and RF, particularly affects small joints of metacarpalia and metatarsalia. Biomechanical characteristics of these joints can have an influence on the local microcirculation and protein metabolism (McInnes et al., 2011). Straub et al. (Straub, 2007, 2014) proprose a close connection between the onset and course of inflammation as well as the energy allocation. In short-term inflammatory conditions the systemic energy allocation shifts to the immune system. In chronic inflammatory conditions, the energy regulation becomes impaired by means of permanent high levels of pro-inflammatory cytokines and high activity of the sympathetic nerve system and hypothalamic-pituitary-adrenal axis with high cortisol level (Straub et al., 2010). The imbalance of the energy expenditure pathways contributes to common systemic features of RA such as diabetes mellitus and insulin resistance or the development of obesity and muscle wasting, since these neurohumoral pathways meet the increased glucose demand for the immune system (Straub et al., 2010). Also, the sympathetic nerve system contributes to the local arthritic activity time-dependently and there-

fore establishes a connection between the central nervous system and the local immune system, in the joint as well as in secondary lymphatic organs (Schaible et al., 2014). It directly exerts a pro-inflammatory effect in early arthritis, but exerts an anti-inflammatory effect in later arthritic stages (Härle et al., 2005). Since sympathetic nerve density in the joint and the lymphoid organs is reduced in late arthritis, the influence of the central nervous system together with its control of energy allocation might become lost in later arthritis and replaced by alternative regulatory mechanisms such as tyrosine-hydroxylase positive cells (Schaible et al., 2014; Pongratz et al., 2013).

2.2.3 Arthritic Phase

The main characteristic of RA is the highly inflamed joint containing high amounts of pro-inflammatory cytokines and tissue-degrading enzymes (Scott et al., 2010). Three parties can be identified as main factors in the development of arthritis: leukocytes, fibroblasts and osteoclasts (Figure 2.3).

The Instigators: Leukocytes Regarding the adaptive immune system, expanding *synovial myeloid* and *dendritic cells* create a T-cell promoting environment and dominance of TH-17 cells, whose secreted IL-17 - together with TNFα - activates fibroblasts, chondrocytes and osteoclasts to produce tissue-degrading enzymes, cytokines such as IL-6, chemokines and growth factors (Miossec et al., 2009; Hot et al., 2011).
Furthermore, autoreactive *T-cells* against citrullinated protein have been found, suggesting that the cellular immune system is directly involved (Cantaert et al., 2009).
B-cells are not only responsible for the production of auto-antibodies, but also for the presentation of the auto-antigen and for cytokine production itself (McInnes et al., 2011).
Macrophages help to sustain inflammation by cytokine expression and antigen presentation. They directly destroy tissue by producing reac-

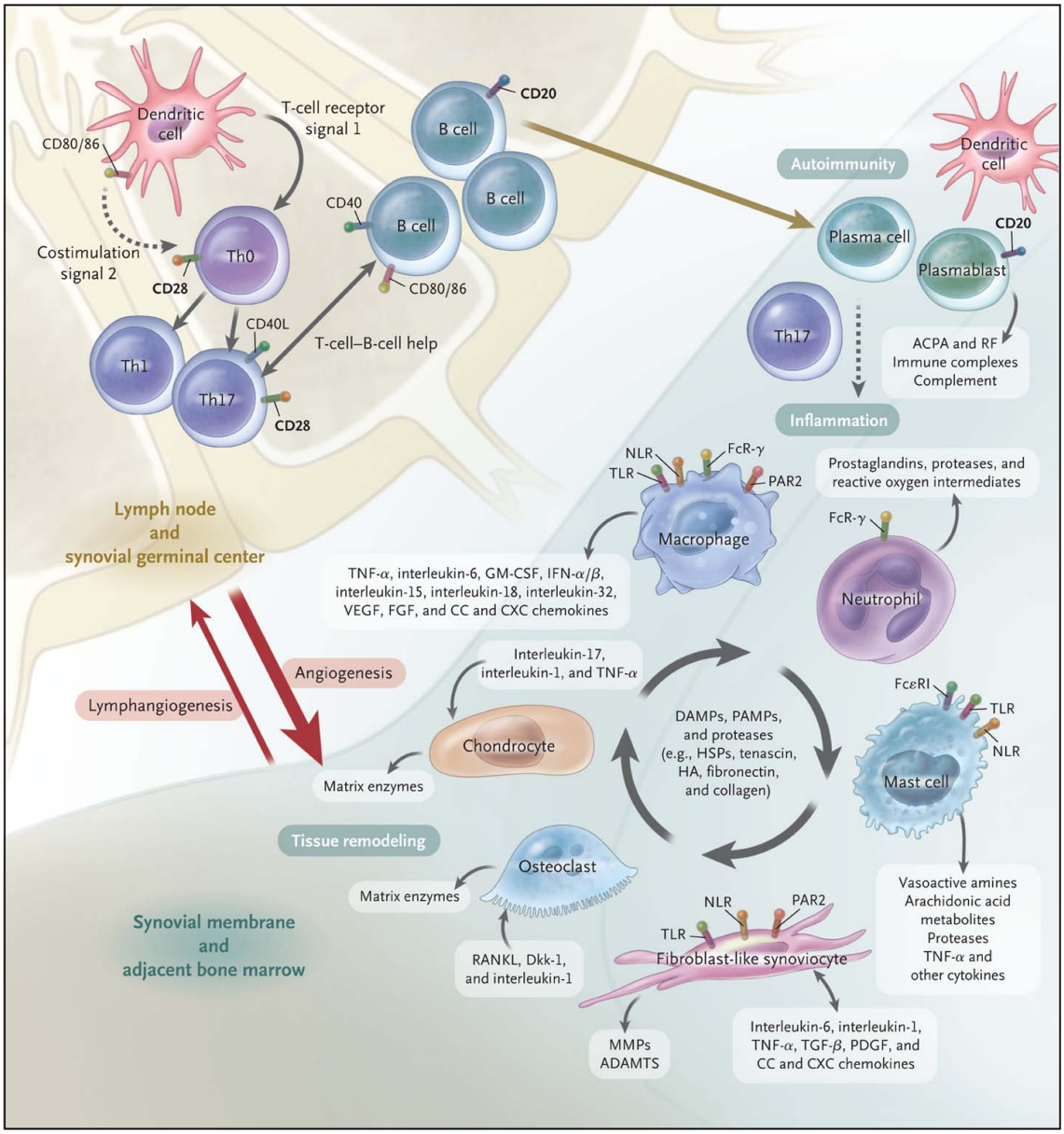

Figure 2.3: Three vital players in Rheumatoid Arthritis. ADAMTS denotes a disintegrin and metalloprotease with thrombospondin-1–like domains, DAMP damage-associated molecular pattern, Dkk-1 dickkopf-1, FcR Fc receptor, FcεRI high-affinity IgE receptor, FGF fibroblast growth factor, GM-CSF granulocyte–macrophage colony-stimulating factor, HA hyaluronan, HSP heat-shock protein, IFN-α/β interferon-α/β, MMP matrix metalloproteinase, NLR nucleotide-binding oligomerization domain–like receptor, PAMP pathogen-associated molecular pattern, PAR2 protease-activated receptor 2, PDGF platelet-derived growth factor, RANKL receptor activator of nuclear factor κB ligand, TGF-β transforming growth factor β, Th0 type 0 helper T cell, Th1 type 1 helper T cell, Th17 type 17 helper T cell, TLR toll-like receptor, TNF-α tumor necrosis factor α, and VEGF vascular endothelial growth factor (McInnes et al., 2011).

tive oxygen intermediates and degrading enzymes (Haringman et al., 2005; McInnes et al., 2011).
Neutrophil Granulocytes are predominant in the synovial fluid, where they support inflammation and sustain cartilage destruction with a host of cytokines, reactive oxygen species and proteases (Eyles et al., 2006; Cornish et al., 2009).
In conclusion, leukocytes generate the highly inflammatory and hypoxic environment that is regarded as the foundation for the development of aggressive fibroblasts.

The Culprits: Fibroblasts The joint capsule and the bone are attached to each other by the synovium, which is organised into a dense lining layer and a loose sublining stroma. Fibroblasts are of mesenchymal origin and tasked with producing and modelling the extracellular matrix of the organic stroma. Thereby, they provide a scaffold for the specialised cells in almost every organ, maintain extracellular homeostasis and integrate stimuli on the microenvironment such as pH reduction or oxygenic stress due to inflammation (Firestein et al., 2012).
The fibroblasts compose the synovial lining without a basement membrane, tight junctions or desmosomes, yet they are riveted together by cadherins such as cadherin-11, and various integrins and receptors of the Ig superfamily, e.g. ICAM and VCAM. These cellular structures play a key role not only in the attachment of the synovial lining, but also in the intracellular signaling and in the regulation of inflammation (Lee et al., 2007; Chang et al., 2011).
In later arthritic stages, the synovial lining undergoes a dramatic invasion of T cells, dendritic cells and B-cells forming germinal centers, and particularly macrophage-like and fibroblast-like synoviocytes (Takemura et al., 2001). All of them migrate, proliferate and increase local joint cellularity by a ten-fold (Firestein et al., 2012). Thereby, the arthritic environment is marked by pro-angiogenic factors and is extensively vascularised. But it remains hypoxic, produces reactive oxygen species and creates chronic oxidative stress on resident fibrob-

lasts (Taylor et al., 2005).
While the synovitis unfolds, Firestein (Firestein, 2003) suggest a direct interaction of macrophage-like synoviocytes and fibroblast-like synoviocytes under the regulation of primarily T-cells. This interaction has turned out to be more complex, since the innate immune system and adjacent bone and cartilage cells are involved as well. The initial site and onset mode of joint arthritis are still unknown.
The persistently inflamed and hypoxic environment changes the synoviocytes, which in turn proliferate and amass a structure called pannus, at which cartilage, ligaments and bone masses are progressively damaged.

The Masons: Osteoclasts Two theories were prompted by Schett and Firestein on the topic of fibroblast-osteoclast relationships (Schett et al., 2010). Depending on the first affected site, synovial inflammation can spread to the bone marrow and induce osteitis. Otherwise, primary osteitis can expand to the joint space and induce synovitis. Kleyer et al. (Kleyer et al., 2014) have coined these differing concepts as the *hen-egg dilemma*. Accordingly, further research is required to discern the prearthritic phase as local or systemic.

2.2.4 Conclusion: Importance of Fibroblasts

In conclusion, fibroblast-like synoviocytes play a vital role in the development and progression of rheumatoid arthritis and are the key players in local joint and bone destruction, alongside the immune system. Although RA is an autoimmune disease, not only should the modulation and suppression of leukocytic activity be considered for therapeutic intervention, but, eventually, decreasing the activity of fibroblasts and osteoclasts may be key to an effective therapeutic strategy.

2.3 The Role of Synoviocytes in Rheumatoid Arthritis

2.3.1 Training the Bad Boy: Proliferation, Cell Death and Destruction

Fibroblast-like synoviocytes are also called synovial fibroblasts (SF) and are key factors in the maintenance of local inflammation as well as in the destruction of adjacent bone and cartilage structures.
Whereas macrophage-like synoviocytes produce a range of pro-inflammatory cytokines, chemokines and growth factors, fibroblast-like synoviocytes respond with the secretion of their own host of cytokines, such as IL-6, and matrix degrading enzymes. The autocrine and paracrine circle sustains inflammation and fosters proliferation of synovial tissue to a pannus at the cartilage-bone borders (Figure 2.3 and 2.4).
In the course of arthritis, fibroblast-like synoviocytes respond to the highly inflammatory environment by a changed behaviour in gene expression and cell characteristics. Effectively, they are trained to be *imprinted aggressors* (Bottini et al., 2013).
The pannus is composed of macrophages, osteoclasts and fibroblast-like synoviocytes and shows characteristics of a local invasive tumour with ongoing destruction of *cartilage surface* by fibroblast-like synoviocytes and *bone surface* by osteoclasts (Figure 2.3).

The Boot Camp: Inflammatory Synovia Fibroblast-like synoviocytes play the central role in sustenance of inflammation and in bone and cartilage destruction (Lee et al., 2007). They are part of the cytokine cycle and are one of the essential sources of IL-6 in arthritis (Nishimoto et al., 2004).
IL-6 and TNFα, as well, are pleiotropic cytokines that moderate the immunological response in inflammation, haematopoesis and oncological diseases (Kishimoto, 1989; Nishimoto et al., 2000). IL-6 mediates between endothelial cells and SF, which, in turn, induce greater neutrophil recruitment in the synovia (Lally et al., 2005). SF are

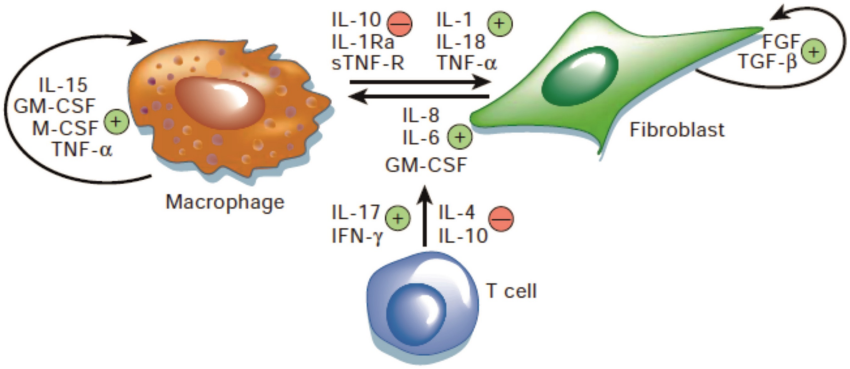

Figure 2.4: Autocrine and Paracrine Cytokine Network in the Synovial Layer. FGF denotes fibroblast growth factor, GM-CSF granulocyte macrophage colony-stimulating factor, IFN-γ interferon γ, IL interleukin, IL-1Ra interleukin 1 receptor antagonist, M-CSF macrophage colony-stimulating factor, sTNF-R soluble tumor necrosis factor receptor, TGF-β transforming growth factor β and TNF-α tumor necrosis factor α (Firestein, 2003).

responsible for the chemoattraction of monocytes by means of CCL2 (MCP-1) secretion (Villiger et al., 1992) and for T-cell recruitment by means of CCL5 secretion (Patel et al., 2001).
These properties reflect their physiological role in the selection and recruitment of leukocytes and in the survival and differentiation of, for example, B cells, which are dependent on IL-6 stimulation by fibroblasts in their immature stage (Firestein et al., 2012).
Fibroblasts are paramount in the prolonged survival and retention of leukocytes, as they produce Interferon-α and Interferon-β (Buckley et al., 2001), CXCL12 and its receptor CXCR4 (Burger et al., 2001; Ohata et al., 2005), CXCL13 and its receptor CXCR5 (Amft et al., 2001) and likely other constitutive chemokines for lymphoid aggregation and organisation in the synovial sublining. In essence, they are not only trainees in the inflammatory boot camp, but they also train immune cells to establish synovitis.

Lesson One: Survive and Spread Cell proliferation and cell death are crucial for developmental and homeostatic processes. In recent years, the range of possible modes of cell death has dramatically expanded. The two most important modes are apoptosis and necrosis. Apoptosis is characterised by a regulated deconstruction of the cell, resulting in

nuclear condensation and fragmentation, followed by scattering of the cell (Mariño et al., 2014). On the other hand, necrosis is marked by cellular leakage and swelling (Vanden Berghe et al., 2014). These two forms cannot be distinctively separated, since other forms of cell death such as necroptosis, a more regulated form of necrosis, have been discovered (Vanden Berghe et al., 2014).
The apoptotic cell death is induced by either extrinsic or intrinsic factors (Figure 2.5). The CD95 (Fas) ligand binds to its receptor, CD95, and activates several proteins, which in turn cleave the aspartat protease pro-caspase 8 into its active form. In a similar mechanism TNF binds to the TNF-related apoptosis-inducing ligand (TRAIL) receptor, which also activates caspase 8. Caspase 8 triggers and orchestrates further caspases and causes the mitochondrial outer membrane permeabilization (MOMP). The mitochondrial leakage releases cytochrome C, which is responsible for the assembly of caspase 9 to the apoptosome. MOMP is directly induced by cellular stress, e.g. nitroxic radicals or radiation.
Though fibroblast-like synoviocytes in RA are under inflammatory, hypoxic and nitrogenic stress, they show a reduced sensitivity to apoptosis. This is because chronic inflammation, in particular, changes behaviour of SF in the intimal synovial lining to a tumorous character:

Bcl-2 and mcl-2 expression increases, which serves as an anti-apoptotic signal (Matsumoto et al., 1996; Perlman et al., 2000; Liu et al., 2005).

CD95/Fas is widely expressed in RA synovia, although SF show a resistance to CD95 treatment (Imamura et al., 1998). This resistance continues *in vitro* (Meinecke et al., 2007).

P53 undergoes somatic mutation similar to many tumours (Firestein et al., 1997; Yamanishi et al., 2002).

In sum, somatic mutation of genetic content and epigenetic modulation of gene expression might explain certain responses by inflam-

matory signals in rheumatoid arthritis synovial fibroblasts (RASF), different from osteoarthritis synovial fibroblasts (OASF) or healthy control samples. Their resistance to apoptosis also changes the proliferative behaviour of SF. The morphological hallmark of this behaviour is the pannus.

Lesson Two: Invade and Infiltrate The pannus can be seen as the forefront of invasion into neighbouring structures, such as cartilage and bone. Fibroblast-like synoviocytes evade contact inhibition and substantially invade and destroy cartilage (Müller-Ladner et al., 1996), even beyond distances similar to metastasation (Lefèvre et al., 2009). The degree of fibroblast invasiveness correlates with the radiographic stage of joint destruction (Tolboom et al., 2005). Therefore, alteration of the invasive character of RASF is another step in the pathogenesis of RA.

Lesson Three: Destroy and Decay SF are crucial in the destruction of joint cartilage, as they produce degrading matrix metalloproteinases (MMP), of which

the secretable collagenases MMP-1, -8, -13;

the non-secretable membrane-anchored collagenases MMP-14 and -16 and

the stromelysins MMP-3 and -10

are mainly responsible for matrix degradation (Bartok et al., 2010; McInnes et al., 2011; Firestein et al., 2012).

The secretion is triggered by cytokines including TNFα, TGFβ or IL-1β. IL-1β is the strongest inducer of MMP secretion via activation of MAPK/AP-1, STAT and NFκB pathways (Li et al., 2001; Mengshol et al., 2000; Brauchle et al., 2000; Barchowsky et al., 2000). Therefore, the reduction of MMP secretion by SF should be a main therapeutic target in RA. This might be achieved by a reduced secretion of these cytokines or by a reduced activation of intracellular signaling.

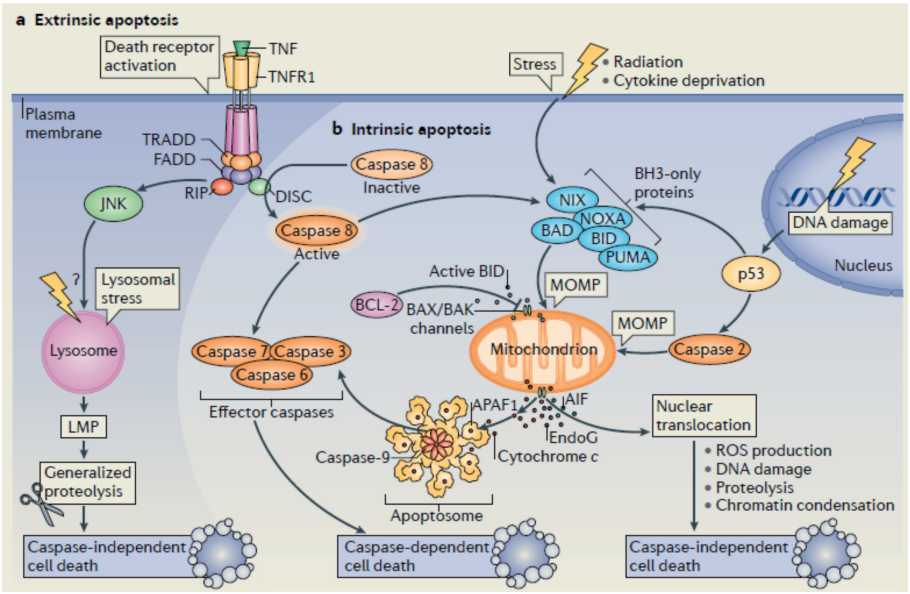

Figure 2.5: The extrinsic and intrinsic mechanism of apoptosis. AIF denotes apoptosis-inducing factor, APAF-1 apoptotic protease-activating factor1, BAK BCL-2 antagonist or killer, BAX BCL-2-associated X protein, BCL-2 b-cell lymphoma protein 2, BH3 BCL-2 homology 3, BID BH3-interacting domain death agonist, CD95 cluster of differentiation 95 or Fas receptor, DISC death-inducing signalling complex, DNA deoxyribonucleic acid, EndoG endonuclease G, FADD FAS-associated death domain, JNK JUN N-terminal kinase, MLMP lysosomal membrane permeabilization, MOMP mitochondrial outer membrane permeabilization , NIX NIP3-like protein X, PUMA and NOXA p53 upregulated modulator of apoptosis, RIP receptor-interacting protein, ROS reactive oxygen species, TNFR1 tumour necrosis factor receptor 1, TRADD TNFR1-associated death domain and TRAILR TNF-related apoptosis inducing ligand receptor (Mariño et al., 2014).

2.3.2 The Inner Ambition: MAPK, CREB and Their Role in Survival, Invasion and Destruction

MAPK In RA fibroblasts, the intracellular system of mitogen-activated protein kinases is the most widely studied signaling pathway and vital for the secretion of MMPs and inflammatory cytokines (Bottini et al., 2013). This highly conserved system consists of three distinct, but not completely separate, MAPK pathways: ERK, p38 and JNK (Figure 2.6) (Widmann et al., 1999).
After four levels of phosphorylation and activation of distinct kinases, phospholipases and cytosceletal proteins (Johnson et al., 2002), the kinases ERK, p38 and JNK enter the nucleus and activate transcription factors. By this means of shaping gene expression, ERK is mainly associated with cell proliferation and differentiation, whereas p38 and

JNK are involved in the cellular response to various forms of stress (Johnson et al., 2002). All three signaling pathways show higher activity in RASF compared to OASF (Han et al., 1999; Schett et al., 2000), which might result from chronic inflammation and stress, but also from epigenetic changes in the genes of these proteins (Bottini et al., 2013).

ERK Signaling At least in part, ERK is activated by TNF in RASF, promotes proliferation and apoptosis (Görtz et al., 2005). Furthermore, activation of ERK fosters the secretion of IL-6, IL-8 and MMP-3 (Calmon-Hamaty et al., 2011). The ERK pathway has also been shown to mediate IL-6 secretion after IL-1 stimulation of RASF (Dou et al., 2013).

JNK Signaling MMP secretion is mainly regulated by the MKK7-JNK-AP1 signaling axis (Bartok et al., 2010). Upon IL-1 stimulation, the kinase MKK7 is able to phosphorylate JNK, which itself activates c-JUN. Phopshorylated c-JUN binds to the FBJ murine osteosarcoma viral oncogene homolog (FOS) and complements the AP1 transcription factor complex, which binds to specific MMP gene loci in RASF (Bottini et al., 2013). This is the reason why the secretion of the important MMP-1, -3 and -13 are dependent on the active JNK pathway (Han et al., 2001). JNK shows a higher activation upon stimulation with IL-1 in RASF, compared to OASF (Han et al., 1999).

p38 Signaling In addition to JNK, p38 is the pivotal regulator for fibroblast aggressiveness. Blocking the p38 pathway in RASF has revealed their involvement in MMP-1 and -3 production as well as IL-6 and -8 secretion upon IL-1 stimulation (Westra et al., 2004; Inoue et al., 2005, 2006). The therapy of RA with p38 inhibitors did not show greater benefit compared with current regimens (Damjanov et al., 2009; Genovese et al., 2011). This proves the complex and redundant role of the p38 signaling pathway, while heterotropic and even opposed

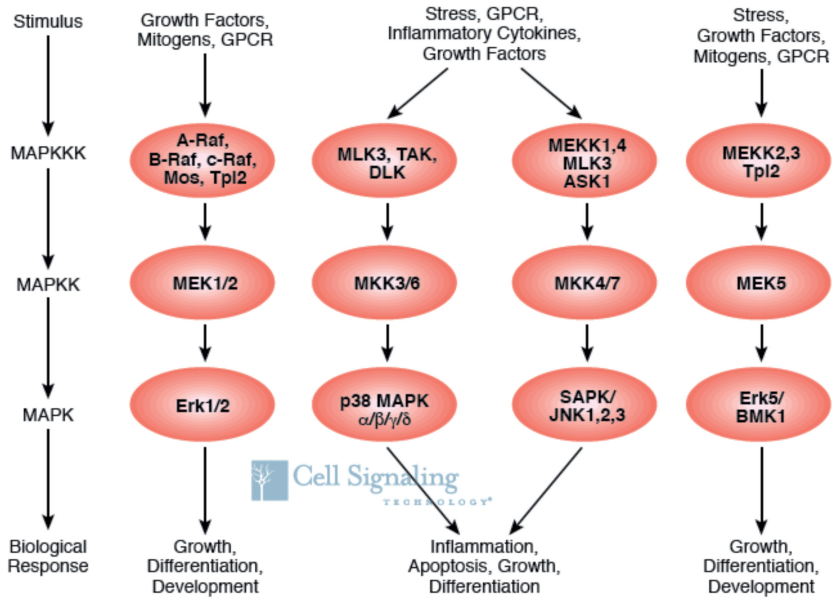

Figure 2.6: Overview of the MAPK system. ASK 1 denotes apoptosis signal-regulating kinase 1, BMK big MAPK kinase, DLK1 delta-like 1, ERK extracellular-signal regulated kinase, GPCR G protein-coupled receptor, JNK c-Jun N-terminal kinase, MAPK mitogen-activated protein kinase, MAPKK mitogen-activated protein kinase kinase, MAPKKK mitogen-activated protein kinase kinase kinase, MEK MAPK/ERK kinase, MEKK MAPK/ERK kinase kinase, MKK p38 MAPK kinase, MLK mixed-lineage kinase, Mos V-mos Moloney murine sarcoma viral oncogene homolog, p38 protein 38, Raf rat fibrosarcoma kinase, SAPK stress-activated protein kinase, TAK TGFβ-activated kinase and Tpl2 tumor progression locus 2 (Cell Signaling Technology, 2010).

function in fibroblasts and leukocytes have to be considered (McInnes et al., 2011; Bottini et al., 2013).

CREB pathway Another mechanism of altering gene expression by extracellular stimuli is the cAMP-responsive element binding protein (CREB). Upon stimulation by catecholamines, intracellular cyclic AMP increases and protein kinase A is activated, which then phosphorylates CREB (Gonzalez et al., 1989). The MAPK ERK2 and p38 also are able to enhance CREB activity through the activation of mitogen- and stress-activated protein kinase MSK (Deak et al., 1998; Tan et al., 1996) and pp90(RSK) family RSK2 (Xing et al., 1996). As a result, these MAPK and CREB are able to induce IL-6 secretion after IL-1 stimulation in RASF (Dou et al., 2013). Another mechanism of

CREB activation is the Ca2+ influx mediated by Ca2+/calmodulin-dependent kinases (Dash et al., 1991).
The active CREB binds the CREB-binding protein in the nucleus (Chrivia et al., 1993) and changes the expression of hundreds of genes affecting signaling, metabolism and proliferation (Impey et al., 2004). Particularly in RASF, evidence of the role of the CREB pathway is rare. For example, phosphorylated CREB has been found to be higher in RASF than in OASF (Pérez-García et al., 2011) and to be functionally involved in the IL-6 secretion by RASF (Ishizu et al., 2010).

2.3.3 Conclusion: Synoviocytes as a Therapeutic Target

The current success of biologicals in the treatment of RA proves that the modulation of the immunological process in rheumatoid arthritis is crucial for breaking the inflammatory cycle in the joint, but this might be not sufficient. Since synovial fibroblasts in RA show independent aggressive behaviour apart from immunological regulation, targeting them might be an effective adjuvant therapy. For example, Lee et al. (Lee et al., 2007) showed the anti-inflammatory effect of a novel non-immunological antibody against cadherin-11, an important building block for the synovial layer and regulator of inflammatory responses in RASF (Chang et al., 2011). As Bottini et al. (Bottini et al., 2013) have noted, combination therapies with dual suppression both immunological cells *and* fibroblasts pave the way for innovative treatments. As one possibility, cannabinoids might offer such promising combinations.

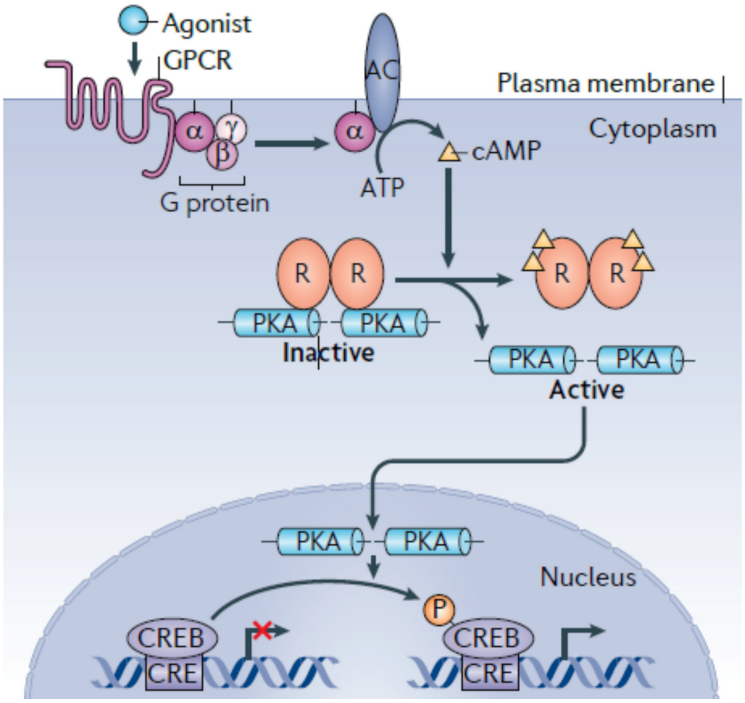

Figure 2.7: Overview of the PKA-CREB-Pathway. AC denotes adenylyl cyclase, cAMP cyclic AMP, CRE/CREB cAMP-responsive element (CRE)-binding protein, GPCR G protein-coupled receptors, PKA protein kinase A and R regulatory subunits of PKA (Altarejos et al., 2011).

2.4 The Endocannabinoid System

2.4.1 The Cannabinoid Receptors

The Cannabinoid Receptors Cannabinoid Receptors CB_1 and CB_2 are G-protein coupled receptors, which, upon activation, induce the second messenger $G_{i/o}$ protein to inhibit adenylate cyclase and to stimulate MAP-Kinases. (Alexander et al., 2011; Pertwee et al., 2010). Cannabis research has been undertaken since the 19th century, when the psychoactive ingredients of cannabis were studied with interest (Iversen, 1999). The chemical synthesis of Δ9-tetrahydrocannabinol by Gaoni and Mechoulam was the gateway to serious scientific investigation of the cannabinoids (Mechoulam et al., 1965).
Cannabis sativa contains over 60 substances, from which Δ9-tetrahydrocannabinol are mostly responsible for psychoactivity (Mechou-

LAM et al., 1970). The synthesis of Δ9-tetrahydrocannabinol analogues allowed for research in the molecular structure and physiological function of cannabinoids, e.g. in psychomotoric vigilance or body temperature. This research eventually led to the relevation of the untilthen orphan receptor SKR6 to be the G-protein-coupled cannabinoid CB_1 receptor (GÉRARD et al., 1990).
Subsequently, a second G-protein coupled cannabinoid receptor, CB_2, was identified in the human promyelocytic leukaemic cell line HL 60 (MUNRO et al., 1993).

Non-Cannabinoid-Receptors: GPR55 and TRPV Besides the classical CB-1 and -2 receptor, the endocannabinoid system also interact with the G protein-coupled receptor 55 (GPR55) and the transient receptor potential channels (TRP) such as for the vanilloid family TRP**V** (ALEXANDER et al., 2011). GPR55 belongs to the GPCR superfamily like CB_1 and CB_2, but transmits its signal to G_q, G_{12} or G_{13} (RYBERG et al., 2007; SHARIR et al., 2010; ALEXANDER et al., 2011), which generally activates downstream proteins like the GTPase Ras homolog gene family member A (RhoA) or phospholipase C. GPR55 has been reported to be involved in inflammatory pain and bone physiology (SHARIR et al., 2010).
The TRPV receptors are divided into the two groups of thermosensitive non-selective cation channels TRPV1-4 and the calcium channels TRPV5 and 6 (ALEXANDER et al., 2011). Classical functions of TRPV include pain generation by heat (STAROWICZ et al., 2007) and regulation of the innate immune system, such as phagocytosis in macrophages (LINK et al., 2010).

2.4.2 The Endogenous Ligands

The identification of receptors for tetrahydrocannabinol suggested endogenous ligands for these receptors. So far, two major endocannabinoids have been identified: 2-arachidonylethanolamide (anandamide or AEA) and 2-arachidonylglycerol (2-AG) (DEVANE et al., 1992; ME-

CHOULAM et al., 1995). In general, these two agonists and the CB_1 and CB_2 receptor constitute the endogenous cannabinoid system (ECS).

2.4.3 General Effects

Since the discovery of AEA and 2-AG, the ECS has revealed to be a highly complex lipid system with distinct effects on neuronal and non-neuronal cells.
Generally, the CB_1 receptor is mainly expressed in the central nervous system, whereas the CB_2 receptor is widely expressed in immune cells (PERTWEE et al., 2010). Changes in the activity of the endocannabinoid system have been shown to correlate with

neuronal conditions: pain and inflammation (HOHMANN et al., 2006; JHAVERI et al., 2007); neurological and neuropsychiatric conditions (BISOGNO et al., 2007).

non-neuronal conditions: immunological (autoimmune and allergic) disorders (OKA et al., 2006); cancer (BIFULCO et al., 2007); and gastrointestinal (STORR et al., 2007) and hepatic (MALLAT et al., 2007) disorders.

interconnected conditions: obesity, metabolic syndrome (MATIAS et al., 2007) and cardiovascular disorders.

However, there have been contradictory results concerning the beneficial or detrimental effects of the endocannabinoids in the same tissue under a given pathological stress, which demonstrates the different interactions of the endocannabinoid system and the complexity of regulation (DI MARZO et al., 2007; PACHER et al., 2006).
Considering effects on rheumatoid arthritis, evidence for a specific mode of action is rare. However, adjacent research fields of inflammation and pain might provide some clue of the endocannabinoid influence on RA. Thus, this introduction only presents the effect of endocannabinoids on inflammation and inflammatory pain in neuronal and non-neuronal systems.

Neuronal Effects: Inflammation and Pain When stressed by inflammation or irritants, levels of endocannabinoids in rodent skin, peripheral nerves and spinal cord are elevated (Oka et al., 2006). Neuropathic pain also increases these levels (Mitrirattanakul et al., 2006; Petrosino et al., 2007), where endocannabinoids serve as analgetic (Agarwal et al., 2007). These features lead to a recent interest in the use of cannabinoids to treat multiple sclerosis.
Though less expressed in the brain, CB_2 is upregulated in microglial and glial cells in response to blood-brain barrier disruption or other inflammatory conditions (Lunn et al., 2008).

Non-Neuronal Effects: Inflammation and Cancer In gastrointestinal inflammation, the ECS is involved in visceral perception and gut motility, but also in inflammation and endothelial damage, since it ameliorates inflammation through CB_1 (Di Marzo et al., 2006, 2007; Massa et al., 2004; D'argenio et al., 2006). In the skin, the endocannabinoid system is able to alleviate contact dermatitis in mice (Karsak et al., 2007).
Endocannabinoids exert anticarcinogenetic effects in a variety of tissues through both CB_1 and CB_2 receptors by modulating a host of different mechanisms such as mitosis, apoptosis, angiogenesis, migration and metastasis (Guzmán, 2003; Bifulco et al., 2007). Yet, CB_1 antagonism with rimonabant also inhibits carcinogenesis (Sarnataro et al., 2006).

2.4.4 Conclusion: Using Complexity

In conclusion, cannabinoids generally exert an anti-inflammatory effect and are likely to be up-regulated in inflamed tissues. However, predictability of agonising or antagonising of the ECS in a given inflamed tissue seems to be very limited. Also, given the difference between early and late inflammation and between murine and human physiology, beneficial effects of endocannabinoids might differ greatly.

2.5 Conclusion: Synergy is Energy

While the therapeutic outcome has significantly improved due to biological treatment, current ambitions aim to combine conventional therapeutics and to decrease cost intensity (Firestein et al., 2012; O'Dell et al., 2013; Sokka et al., 2013). Instead of using single treatments, the usage of synergic factors in a combined therapy might be a powerful strategy to reduce dosage and adverse effects or to widen the therapeutic spectrum of established drugs, but demands appropriate selection of therapeutics. The different points of action of the therapeutics and their interactions with each other have to be considered. Selection of the proper treatment for a particular patient will be crucial to stay ahead of the forthcoming pressure on treatment costs. There is increasing evidence that cannabinoid treatment might be useful to be integrated in such a supportive strategy.

2.6 Purpose and Goal: From Pathogenesis to Treatment

Evidence of the influence of endocannabinoids on intracellular signaling of the fibroblast-like synoviocytes and its consequences on proliferation and cell death is still limited. However, this is crucial for

1. a further understanding of the pathogenic influence of the endocannabinoids in RASF and its role in RA, and thus
2. the development of potential new or combined therapies with established cannabinoid agonists or antagonists.

Therefore, this study investigated the influence of selective cannabinoid agonists and the endogenous agent anandamide on two of the main mechanisms in fibroblasts, namely intracellular MAPK and CREB signaling, which coordinate proliferation, cytokine secretion and apoptosis.

Materials and Methods

Table 3.1: Disposable Materials

Material	Supplier
Culture flasks	Corning, New York, USA
Multiflask	BD Falcon, Plymouth, UK
Safe Lock Tubes	Eppendorf, Hamburg, Germany

Table 3.2: Technical Devices

Material	Supplier
Scepter™ Cell Counter	Merck Millipore, Billerica, USA
iMark™ Microplate Reader (for Absorbance)	Bio-Rad, Hercules, USA
ChemiDoc™ XRS+ Molecular Imager	
Victor Multilabel Counter	Perkin Elmer, Waltham, USA
Coulter EPICS XLMCL™	Beckmann Coulter, Brea, USA

Table 3.3: Agents and Stimulans

Material	Supplier
Cannabinoids	
Anadamide	Tocris, Bristol, UK
Am251	
JTE	
JNJ	
CP47497	
Hu210	
Cytokines	
TNFα	Peprotech, Rocky Hills, USA
Interleukin-1β	
Lipopolysaccharide	

Table 3.4: Cell Acquisition and Culturing

Material	Supplier
RPMI	Sigma, Deisenhofen, Germany
Amphotericin B 0.25 mg mL^{-1}	
Bovine Serum Albumin	
HEPES-Buffer 1 mol	
Fetal Calf Serum	
Penicilline/Streptomycine	
Trypsin	
PBS	
2-Mercaptoethanol	
Ascorbic acid	
Calcium	
Ammonium chloride	
Ammonium bicarbonate	
DMSO	
L-glutamine 200 mmol	Invitrogen, Carlsbad, USA
Dispase I	Roche Diagnostics, Mannheim, Germany
EDTA	PAA Laboratories, Pasching, Austria

Table 3.5: Intracellular Signaling

Material	Supplier
Antibodies	
Phospho-p44/42 - Erk1/2 on site Thr202/Tyr204	Cell Signaling Technology, Danvers, USA
Anti-p38 (phospho T180 + Y182) Antibody	Abcam, Cambridge, UK
Anti-CREB (phospho S133) Antibody	
Polyclonal Goat Anti-mouse Immunoglobuline HRP	Dako, Glastrup, Denmark
Pierce Goat Anti-Rabbit Poly-HRP	Thermo Scientific, Waltham, USA
Washing, Blocking, and Development	
Triton 100X	AppliChem, Darmstadt, Germany
Bovine serum albumin	Sigma, Deisendorf, Germany
3,3',5,5'-Tetramethylbenzidine Tablet 1mg	
1-Step™ Ultra TMB-ELISA	Thermo Scientific, Waltham, USA
Proteome Profilers	
Proteome Profiler Human Phospho-Kinase Array Kit	R&D Systems, Minneapolis, USA
Proteome Profiler Human Cytokine Array Kit	
Amersham ECL™ Western Blotting Reagents	GE Healthcare, Chalfont St Giles, UK

Table 3.6: Cell Viability and Cytotoxicity

Material	Supplier
Proteome Profiler Apoptosis Array Kit	R&D Systems, Minneapolis, USA
LDH Cytotoxicity Detection Kit	ClonTech, Mountain View, USA
Apoptosis Detection Kit	BD Biosciences, San Jose, USA
Lysis Buffer 6	

3.1 Patients

Cells were acquired from a total of 24 patients with long-term rheumatoid arthritis according to the ACR criteria (Arnett et al., 1988) and 22 patients with osteoarthritis. Every patient underwent an elective operation for knee replacement. All subjects gave informed consent to participate in this study. The study was approved by the Ethics Committee of the University of Regensburg. Relevant clinical data and anti-inflammatory drugs are listed in Table 3.7.

Table 3.7: Characteristics of Patients. CRP denotes c-reactive protein, NSAID nonsteroidal anti-inflammatory drug and DMARD disease-modifying antirheumatic drug

	OA	RA
Age [years]	71 ± 9.4	61 ± 11.3
Sex [numbers of males/females]	10/12	3/21
CRP [mg/L]	4.1 ± 4.6	9.3 ± 9.9
Usage of NSAID [No.]	19	22
Usage of prednisolone [No.]	1	20
Daily dosis of prednisolone [mean ± standard error in mg]	N/A	12.1 ± 6.3
Usage of methotrexate	N/A	9
Usage of DMARD	N/A	13
Usage of biologicals	N/A	3

3.2 Cell Culture

3.2.1 Tissue Preparation

Tissue was extracted directly after opening of the articular capsule and stored in transportation medium, which consists of RPMI-1640 with 25 mM HEPES, 5% Fetal Calf Serum, 1 % penicilline/streptomycin, 30 µM 2-mercaptoethanol, 0.57 mM ascorbine acid and 1.3 mM calcium.
Tissue samples were cut into 9 cm^2 sections and detached from fat tissue or highly vascularised parts. A portion of the samples was stored at −80 °C, and the remaining samples were used for cell culture. For extraction, the tissue samples were smashed and solubilised with Dispase I for 1 h at 37 °C on a rocking platform shaker. The suspension was filtered with 70 µm of pore size and centrifuged for 10 min at 300·g. The pellet was resuspended, incubated with erythrolysis buffer (20.7 mg ammonium chloride, 1.97 mg ammonium bicarbonate, 0.09 mg EDTA, 1 L H_2O) for 5 min and re-centrifuged for 10 min at 300·g. The final pellet was resuspended with RPMI-Medium containing 10 % FCS and $1x10^5$ cells were seeded in 75 cm^2 culture flasks. The culture medium was changed on the second day.

3.2.2 Cryopreservation and Thawing

For long-term storage, cells were cryo-conserved in fluid nitrogen at −196 °C. Prior to this, cells were

1. detached with 2.5 mL EDTA/Trypsin solution for 5 min,
2. centrifuged at 300·g,
3. resuspended in a protection solution against cristallisation and cell lysis (cell culture medium with 40 % FCS and 10 % DMSO), and
4. frozen stepwise, first down to −80 °C for 48 h, then to −196 °C.

Prior to culture, frozen cells were

1. quickly thawed at 37 °C,
2. transfered to 10 mL fresh pre-warmed cell culture medium,
3. centrifuged at 300·g for 10 min,

4. resuspended in fresh pre-warmed medium and
5. finally transferred 1×10^5 in 75 cm^2 culture flask.

The culture medium was changed on the second day.

3.2.3 Cell Culturing

Cells were cultured in culture medium (RPMI-1640 with 1 % HEPES, 5 % Fetal Calf Serum, 1 % penicillin/streptomycin, 2 % L-glutamine, 0.1 % amphotericin B) at a constant temperature of 37 °C and 2 % O_2/5 % CO_2 in a fully saturated environment in 175 cm^2 and 75 cm^2 culture flasks.
Cells were split 1:2 or 1:3 with 10 % trypsin/-PBS solution every week according to their confluence. Only passages 2 to 7 were used for the experiments. This was important since macrophage-like synoviocytes are still present in the first cell culture passage and fibroblast-like synoviocytes degenerate from the 7th passage onwards (Rosengren et al., 2007).

3.3 Detection of Intracelluar Activation of ERK, p38, c-JUN and CREB

Cells were transferred into 96-well plates with 10,000 cells/well. Cells were starved for 24 h in serum-free medium to reduce basal phosphorylation. Thereafter, they were incubated with agents described in Table 3.3. For the quantification of intracellular posphorylated kinases, a cell-based ELISA was performed according to the following procedure:

1. Fixation of cells by formalin (4 %)-PBS solution for 20 min
2. Washing twice with Washing Buffer (PBS, Triton 0.1 %) for 10 min
3. Incubation with Blocking Buffer (Washing Buffer, 1 % BSA) for 1 h.
4. Washing once as described in step 2.
5. Incubation of the first antibody overnight at 4 °C.
6. Washing twice as described in step 2.
7. Incubation of the second antibody for 1 h.

8. Washing twice as described in step 2.
9. Development with TMB
10. Stop reaction with 2M NaSO4.
11. Absorbance was recorded at 450 nm with an iMark™ Microplate Reader.

3.4 LDH Cytoxicity Detection

In late apoptosis and necrosis, cell membrane leakage releases intracellular lactate dehydrogenase. After stimulation in serum-free medium, the supernatant was transferred to a separate 96-well plate and incubated with the Cytotoxicity Detection Kit (ClonTech, USA) acoording to the retailer instructions. Measurement of absorbance was performed at 490 nm with an iMark™ Microplate Reader.

3.5 Flow Cytometry with Annexin V and Propidium Iodide

Annexin V binds to extracellular phospholipid phosphatidylserine, whose translocation from the inner to the outer plasma membrane leaflet occurs during early apoptosis. In late apoptosis and necrosis, membrane stability deteriorates, so that membrane leakage leads to DNA discharge. Cytoplasmatic DNA is detected by propidium iodide (PI).
Cells were detached with Accutase™ and transferred into 1.5 mL Eppendorf Safe Lock Tubes. Cells were incubated with FITC and 7-AAD for 15 min at room temperature. Data acqusition was performed with the flow cytometer Coulter EPICS XLMCL™. Data output was analysed with FlowJo™ (FlowJo LLC, Oregon, USA, V.10). A subpopulation of fibroblasts was set to exclude cell detritus.

3.6 Proteome Profiler: Human Phosphokinase, Apoptosis and Human Cytokine Kit

Cells were cultured in 75 cm^2 culture flask and starved in serum-free medium for 48 h in normoxic or hypoxic (1 % O_2) conditions. Cells were stimulated as described below, in normoxic or hypoxic environ-

ments, respectively. Cells were washed twice with Dulbecco's PBS, solubilised with Lysis Buffer 6, and immediately stored at −25 °C. The Human Phospho-Kinase Kit (R&D Systems, Minneapolis, USA) measures the relative level of phosphorylation of 45 different intracellular kinases. The Human Apoptosis Array Kit measures the expression of 35 apoptosis-related proteins. Both Kits use specific antibodies attached to nitrocellulose slides and are performed as a sandwich enzyme-linked immunosorbent assay ELISA. Preparation and detection of samples were performed according to the instructions provided by the manufacturer. Chemiluminiscence was developed using the reagents included in the kit as well as Amersham ECL™ Western Blotting Reagents. Plots were read with a BioRad ChemiDoc™ XRS+ Molecular Imager, using a defined frame of 13.5 cm x 10 cm and a binning of 3x3 to increase sensitivity without any filtering. Analysis of relative dot density of the photographs was performed with Image-Lab™ (Bio-Lab Laboratories, V. 4.0.1), using the digital mean pixel intensity of the photographs for each point on the slide

3.7 Statistical Analysis

Statistical analysis and plotting were performed with SPSS (V.22, IBM™) and SigmaPlot™ software (V.13, Systat Software Inc.). Each statistical analysis was performed non-parametrically with the Mann-Whitney Rank-Sum test. A p-value <0.05 was the significance level.

Results and Discussion

4.1 The Predominance of ERK

The Proteome Profiler showed the relative phosphorylated levels of most intracellular signaling proteins and revealead ERK to be the most active MAPK in unstimulated RASF, followed by p38, pan-JNK, c-Jun and CREB in normoxia (Figure 4.1). In hypoxia, expression levels changed minimally, whereas pan-JNK was significantly suppressed (ibid.).

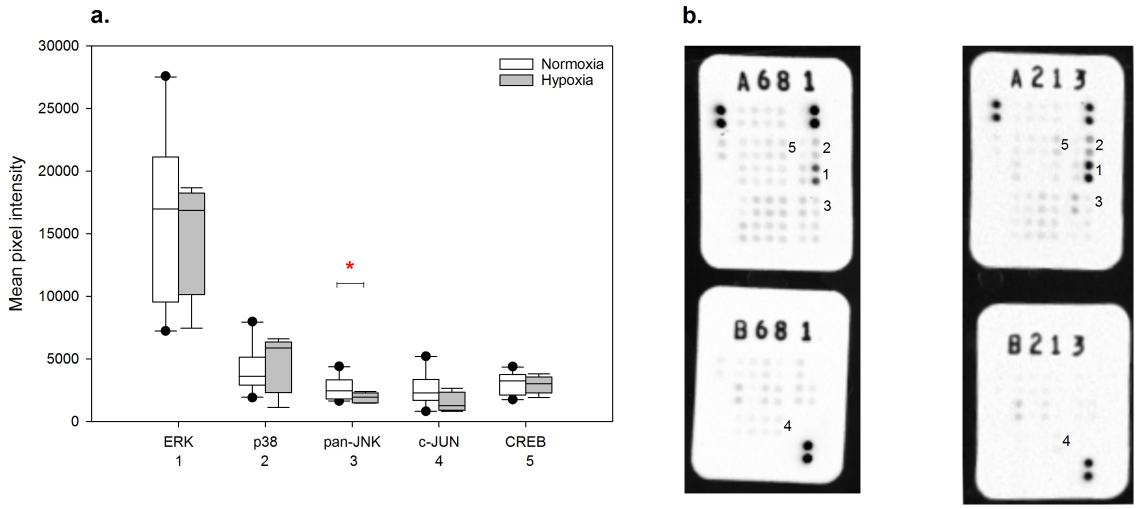

Figure 4.1: Rheumatoid arthritis synovial fibroblasts were incubated in normoxia (n=4) or hypoxia (n=5) for 24 h, lysed and incubated on Proteome Profiler™ membranes. A. Numerical expression of MAP-kinases and CREB on the Proteome Profiler™. ERK denotes extracellular-signal regulated kinase, p38 protein 38 MAPK, Pan-JNK the total amount of c-Jun N-terminal kinase, c-JUN as member of the Jun transcription factors and CREB cAMP-responsive element binding protein. B. Actual Proteome Profiler™ slides after 15 min in normoxia (left) and hypoxia (right). The numbers 1-5 refer to the set of dots left to them and denote the MAPK described in a.*= $p<0.05$.

The stimulation of RASF and OASF for 15 min showed a significantly higher phosphorylation of ERK, p38, CREB and c-JUN in normoxic and hypoxic environment (Figure 4.2). In RASF but not in OASF, the normoxic and hypoxic ERK phosphorylation was significantly higher in response to TNF ($10\,\mathrm{n\,mL^{-1}}$) than to IL-1β ($1\,\mathrm{n\,mL^{-1}}$), whereas the normoxic phophorylation of p38 was significantly higher after stimulation with IL-1β. In contrast, TNFα activated c-JUN more effectively in OASF compared to RASF under hypoxic conditions (Figure 4.2).

4.2 The Antagonistic Effects of CB_1 and CB_2 on p38

Samples were pre-incubated with CB_1 and CB_2 agonists for 5 h and then stimulated with the pro-inflammatory cytokines TNFα, IL-1β and Lipopolysaccharide (LPS) for 15 min. Positive control samples were stimulated only with the cytokines.
Compared to the positive control samples, CB_1 and CB_2 agonists showed significantly different inhibitory and stimulatory patterns on intracellular signaling in normoxia and hypoxia (Figure 4.3 and 4.4). In normoxia, the CB_2 agonist GP1a attenuated the IL-1β-induced phosphorylation of the stress-related kinases p38 and CREB. In OASF, the CB_2 agonist repressed TNFα stimulation of p38 and the LPS-induced phosphorylation of p38 and CREB. In hypoxia, the LPS-induced stimulation of ERK was repressed by CB_2 agonism only in RASF. In OASF, GP1a more extensively repressed TNFα-induced stimulation of p38 as well as the TNFα- and IL-1β-induced stimulation of CREB. The MAPK c-JUN was similarly repressed by CB_1 and CB_2 in both OASF and RASF after TNFα, whereas only the CB_2 agonist reduced c-Jun activation after IL-1β an LPS stimulation both in RASF and OASF, compared to the positive control samples.
In contrast, samples preincubated with CB_1 agonist showed slightly (in OASF) and significantly (in RASF) higher p38 phosphorylation after IL-1β stimulation, compared to the positive control samples in normoxia and hypoxia (Figure 4.3 and 4.4).

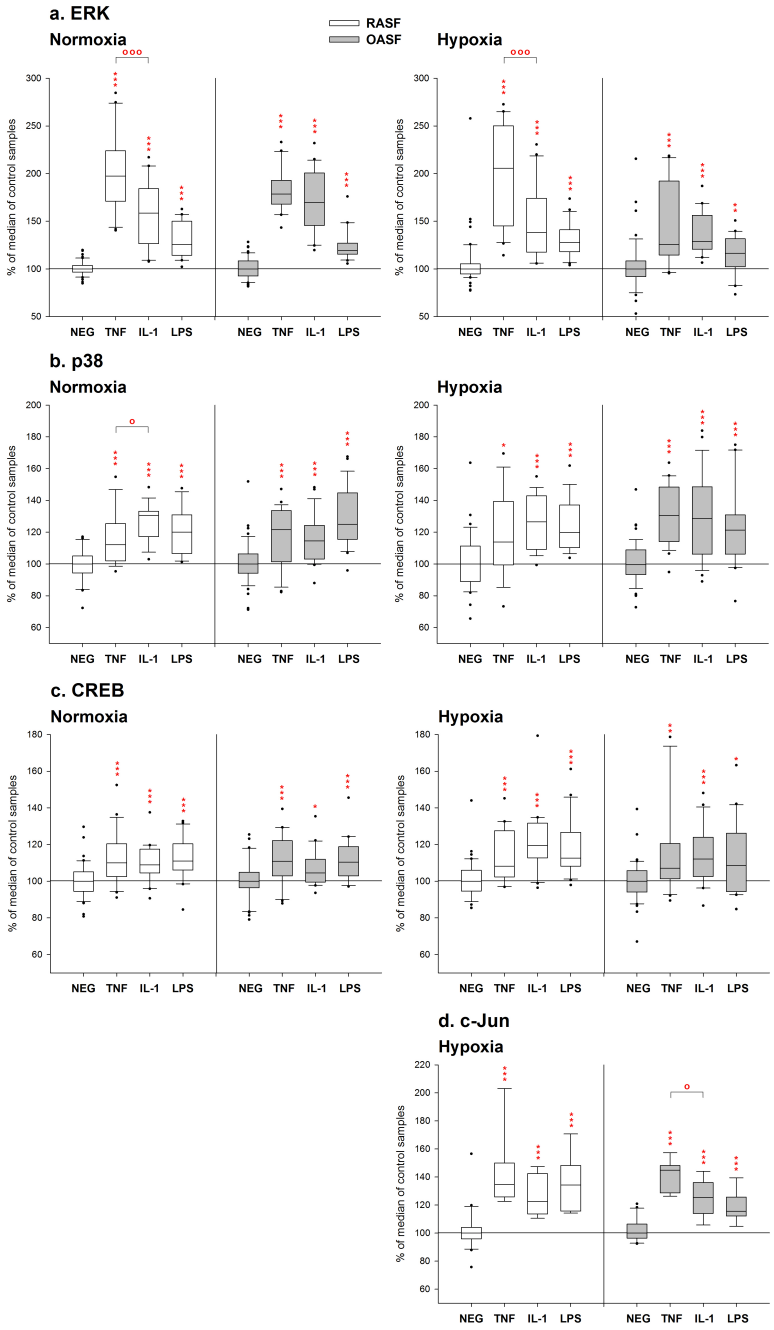

Figure 4.2: Rheumatoid arthritis synovial fibroblast (RASF, n=5, n=2 for c-Jun) and Osteoarthritis synovial fibroblasts (OASF, n=5, n=2 for c-Jun) were stimulated with Tumor Necrosis Factor α (TNFα, 10 ng mL^{-1}), Interleukin-1 (IL-1β, 1 ng mL^{-1}) or Lipopolysaccharide (LPS, 10 µg mL^{-1}) for 15 min. The level of phosphorylation of extracellular-signal regulated kinase (ERK), p38 MAPK (p38), cAMP-responsive element binding protein (CREB) and c-Jun protein (c-JUN) was measured with ELISA. Absorbance of the samples is given in percentage of median of the control samples (NEG) on each plate. Samples were compared to the negative control samples: *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

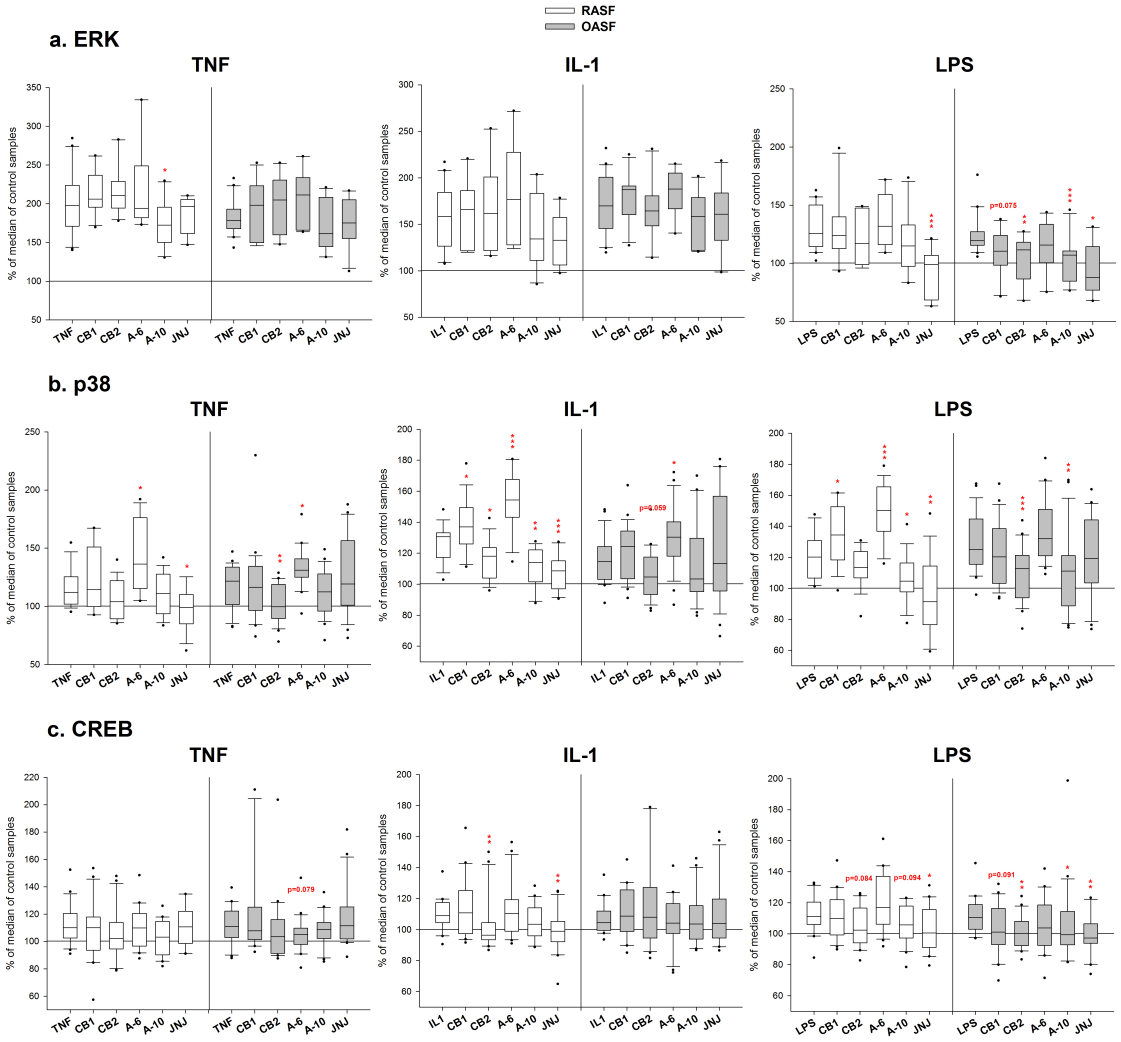

Figure 4.3: Rheumatoid arthritis synovial fibroblast (RASF, n=5) and osteoarthritis synovial fibroblasts (OASF, n=5) were pre-incubated for 5 h in *normoxia* with CB_1 agonist CP 47,497 (CB1, 1×10^{-6} M), CB_2 agonist GP1a (CB2, 1×10^{-7} M), anandamide (A-6, 1×10^{-6} M and A-10, 1×10^{-10} M), or fatty acid amide hydrolase JNJ (JNJ, 1×10^{-7} M), and thereafter stimulated with Tumor Necrosis Factor α (TNF, $10\,\mathrm{ng\,mL^{-1}}$), Interleukin-1 (IL-1, $1\,\mathrm{ng\,mL^{-1}}$) or Lipopolysaccharide (LPS, $10\,\mu\mathrm{g\,mL^{-1}}$). Positive control samples are denoted as their stimulant (TNF, IL-1 or LPS). The level of phosphorylation of extracellular-signal regulated kinase (ERK), p38 MAPK (p38) and cAMP-responsive element binding protein (CREB) was measured with ELISA. Absorbance of the samples is given in percentage of median of negative control samples on each plate. Samples were compared to positive control samples. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, if not mentioned otherwise.

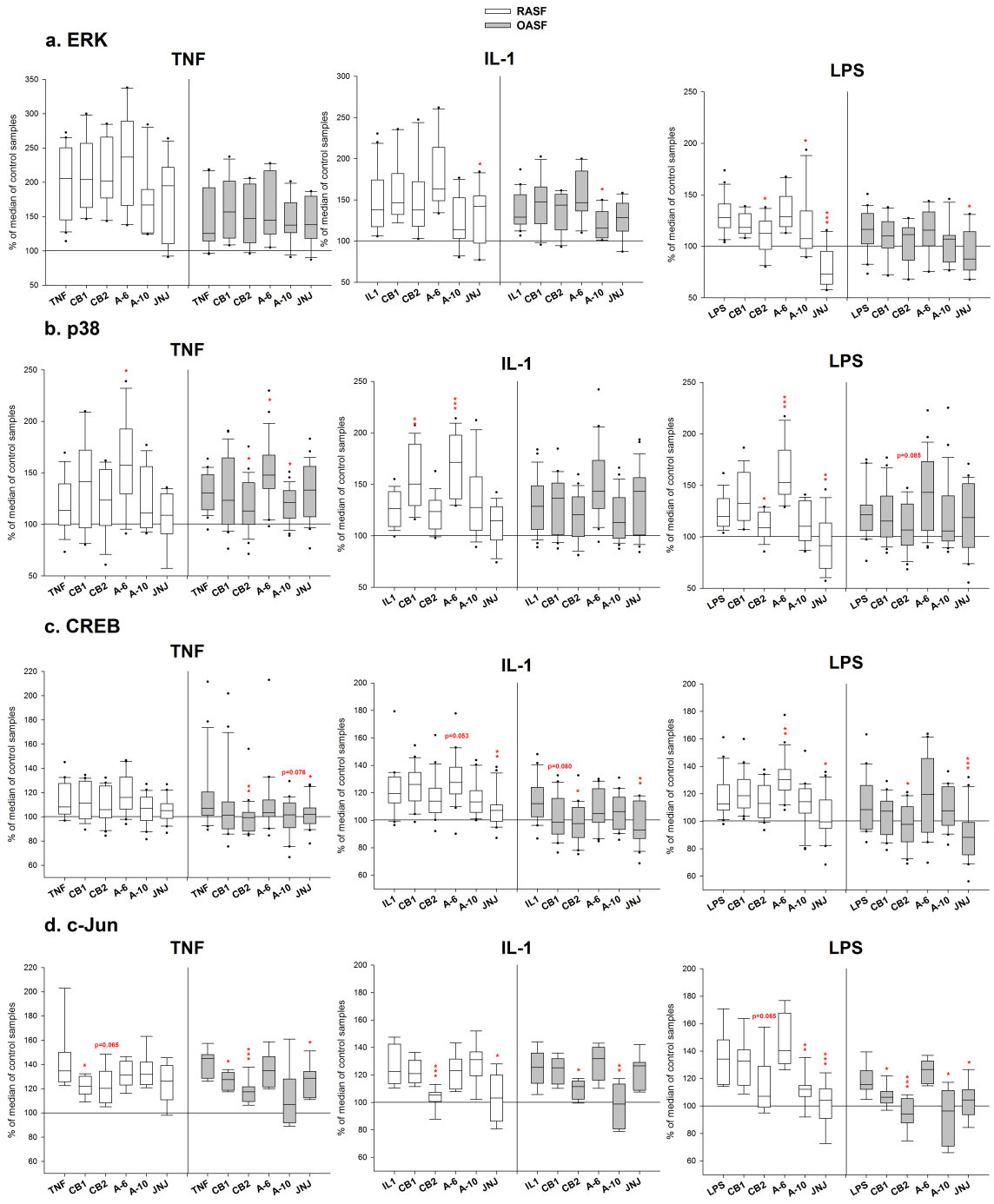

Figure 4.4: Rheumatoid arthritis synovial fibroblast (RASF, n=5) and osteoarthritis synovial fibroblasts (OASF, n=5) were pre-incubated for 5 h in *hypoxia* with CB_1 agonist CP 47,497 (CB1, 1×10^{-6} M), CB_2 agonist GP1a (CB2, 1×10^{-7} M), anandamide (A-6, 1×10^{-6} M and A-10, 1×10^{-10} M), or fatty acid amide hydrolase JNJ (JNJ, 1×10^{-7} M), and thereafter stimulated with Tumor Necrosis Factor α (TNF, 10 ng mL^{-1}), Interleukin-1 (IL-1, 1 ng mL^{-1}) or Lipopolysaccharide (LPS, 10 µg mL^{-1}). Positive control samples are denoted as their stimulant (TNF, IL-1 or LPS). The level of phosphorylation of extracellular-signal regulated kinase (ERK), p38 MAPK (p38), cAMP-responsive element binding protein (CREB) and c-Jun protein (c-JUN) was measured with ELISA. Absorbance of the samples is given in percentage of median of negative control samples on each plate. Samples were compared to positive control samples. *= p<0.05, **= p<0.01, ***= p<0.001, if not mentioned otherwise.

4.3 The Antagonistic Effects of Anandamide

Samples were pre-incubated with anandamide for 5 h and then stimulated with the pro-inflammatory cytokines TNFα, IL-1β and Lipopolysaccharide for 15 min.
In comparison to the positive control samples, anandamide attenuated the cytokine-induced phosphorylation of MAPK at low concentration and increased stimulation at high concentration. Preliminary Proteome Profiler™ experiments revealed a significant decrease in the expression of phosphorylated ERK and pan-JNK after incubation with 1×10^{-7} M AEA in RASF under hypoxia (Figure 4.5). In further experiments using RASF, 1×10^{-10} M AEA repressed the IL-1β- and LPS-induced phosphorylation of p38 in normoxia, and of ERK in hypoxia. In OASF, pre-incubation with 1×10^{-10} M AEA decreased phosphorylation of ERK, p38 and CREB by LPS in normoxia (Figures 4.3 and 4.4). Anandamide at 1×10^{-6} M significantly increased phosphorylation levels of the stress-related kinases p38 and CREB upon TNFα, IL-1β stimulation in OASF and RASF under normoxia, while it enhanced LPS-induced stimulation only in RASF. In hypoxia, however, it increased levels of activated p38 in RASF after incubation with TNFα and IL-1β and in OASF only after incubation with TNFα (Figures 4.3 and 4.4). Furthermore, AEA enhanced IL-1β- and LPS-stimulated CREB activation only in RASF (Figure 4.4).

4.3.1 Blocking the Recycling of AEA with JNJ Decreased MAPK Phosphorylation

JNJ-1661010 (JNJ) is a selective inhibitor of intracellular fatty amide acid hydrolase (FAAH), which leads to increased intracellular levels of anandamide. Samples were pre-incubated with JNJ for 5 h and then stimulated with the pro-inflammatory cytokines TNFα, IL-1β and Lipopolysaccharide (LPS) for 15 min. Experiments with human and rat brain cells showed JNJ to have an IC(50) of 12 nmol on human FAAH with a twofold increase of intracellular anandamide at maxi-

mum (Karbarz et al., 2009). A JNJ dose of 1×10^{-7} M was used to guarantee a maximal inhibition of intracellular FAAH. In comparison to the positive control samples, JNJ exerts an inhibitory effect on MAPK phosphorylation (Figures 4.3 and 4.4). In RASF, FAAH inhibition decreased TNFα-, IL-1β- and LPS-induced phosphorylation of p38 in normoxia and LPS-induced MAPK activation in hypoxia. JNJ decreased activation levels of CREB in RASF and OASF after IL-1β and LPS stimulation, both in normoxia and hypoxia. In OASF, JNJ attenuated LPS-stimulated levels of ERK and CREB in normoxia and TNFα-, IL-1β- and LPS-induced CREB activation in hypoxia.

4.3.2 Repression of Downstream Proteins

The Proteome Profiler also showed anandamide to significantly repress the expression of unstimulated 90 kDa Ribosomal S6 Kinase (RSK) and stimulated Mitogen- and Stress-Activated Protein Kinase (MSK) in hypoxic RASF (Figure 4.5). These two proteins are activated by MAPK ERK and p38 and establish a crosstalk between the MAPK and CREB pathway.

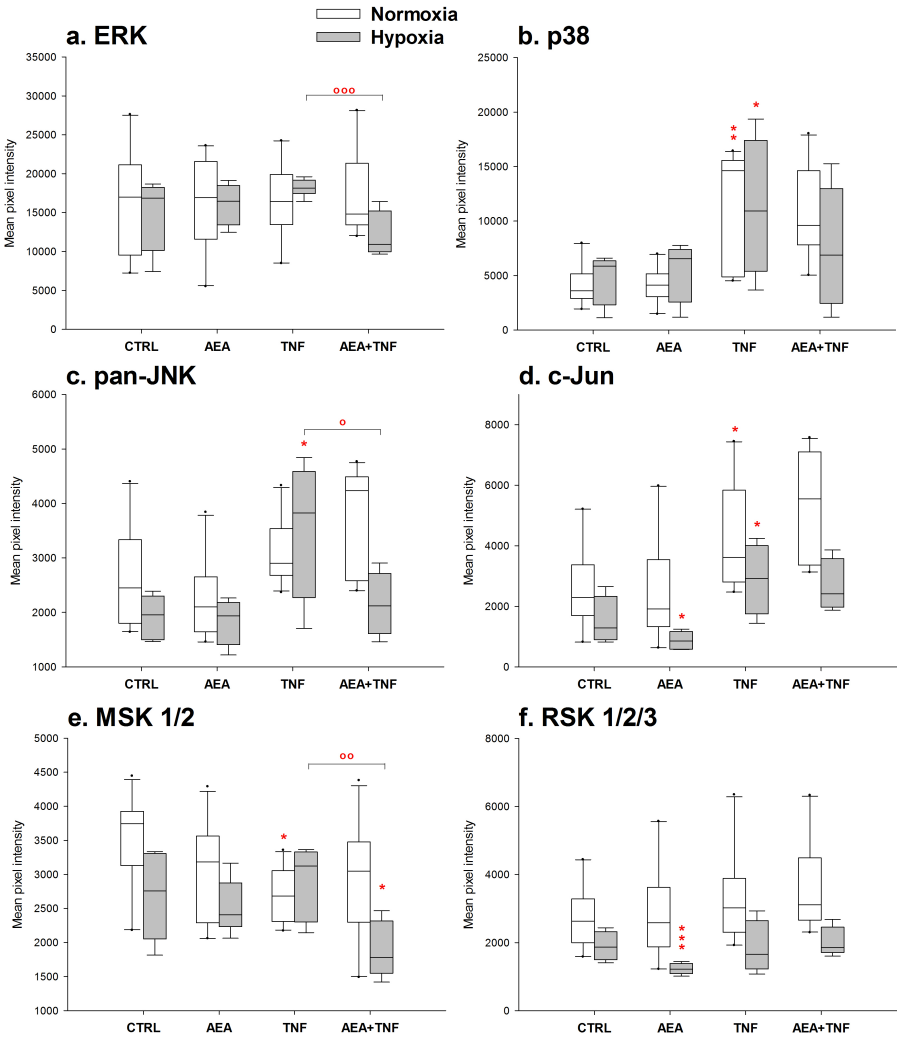

Figure 4.5: Rheumatoid arthritis synovial fibroblast (RASF) were pre-treated with anandamide (AEA, 1×10^{-7} M) for 5 h in normoxia (n=4) or hypoxia (n=5) and subsequently stimulated with TNFα (TNF, 10 ng mL^{-1}). Samples were lysed and incubated on the Proteome Profiler™ slides. Results are given as mean pixel intensities of respective spots of the kinases on the slides after development for 5 min. ERK denotes extracellular-signal regulated kinase, p38 protein 38 MAPK, pan-JNK the total amount of c-Jun N-terminal kinase, c-Jun a member of Jun transcription factors, MSK mitogen- and stress-activated protein kinase and RSK 90 kDa ribosomal S6 kinase. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, vs. negative control sample (CTRL). o= $p<0.05$, oo= $p<0.01$, vs. positive control samples (TNF).

4.4 Conclusion: The Activation Pattern of AEA Resembled CB_1 and CB_2

High concentrations of AEA generally resembled the increase of phosphorylation by the CB_1 agonist CP 47,497, such as in p38 after IL-1β incubation. However, at low concentrations, it attenuated MAPK signaling like the CB_2 agonist GP1a, such as p38 in RASF after incubation with IL-1β or in OASF after incubation with TNFα (Figure 4.3). CB_2 receptors are largely distributed and active peripherally in immune cells and are known to take part in the immunomodulation during the inflammation process. In an inflammatory environment, CB_2 receptors are slightly up-regulated in RASF after stimulation with TNFα, IL-1β or LPS, compared to OASF (Gui et al., 2014). Selective CB_2 agonists attenuated IL-1β-induced p38 and ERK activation in synovial fibroblasts (Gui et al., 2014) and TNFα-induced activation of ERK1/2, p38 and JNK in smooth muscle cells (Rajesh et al., 2008).
The present studies were consistent with these results and furthermore showed for the first time that the endogenous agonist anandamide demonstrated the same anti-inflammatory CB_2-dependent pattern at low concentrations, whereas at high concentrations, it increased phosphorylation, likely through a CB_1-dependent mechanism or through TRPV1 channels. TRPV1 channels are non-selective cation channels. As a consequence, not only intracellular phosphorylation but also membrane depolarisation and calcium homeostasis might be involved in the *in vivo* activation of SF by AEA.
Furthermore, the present studies showed a different suppression and activation pattern of MAP kinases in synovial fibroblasts under normoxia and hypoxia. This insight might give some warranties against possible therapeutic interventions with cannabinoids in RA, since their effects might depend on the local oxygen concentration.

4.5 Activation of MAPK Mainly by CB_1 in Rheumatoid Arthritis Synovial Fibroblasts

4.5.1 Rheumatoid Arthritis Synovial Fibroblasts Are More Prone to Stimulation

After 30 min of incubation with CB_1 agonist CP 47,497, CB_2 agonist GP1a or AEA under normoxia, phosphorylation of ERK were significantly increased in RASF compared to OASF (Figure 4.6). In hypoxia, CB_1 agonism, CB_2 agonism and AEA activated p38 and CREB only in RASF and not in OASF, with the exception of the normoxic activation of CREB by AEA at 1×10^{-8} M. Also in RASF and not in OASF, ERK activation by AEA and GP1a was significantly increased under normoxia compared to hypoxia (Figure 4.6).

4.5.2 The CB_2 Agonist Activated ERK and CREB only in Rheumatoid Arthritis Synovial Fibroblasts

When compared to the negative control samples, the CB_2 agonist GP1a induced a significant ERK and CREB activation only in RASF (Figure 4.6). In addition, the ERK phosphorylation by AEA was significant lower after pre-incubation with the inverse CB_2 agonist JTE-907 than without pre-incubation, whereas CREB activity after stimulation by AEA did not change with or without pretreatment with JTE-907 (Figure 4.7). Generally in OASF, pretreatment with JTE-907 did not change the activation levels of ERK, p38 or CREB after stimulation with CB_1, CB_2 and AEA, compared to unpretreated samples (Figure 4.7).

4.5.3 The CB_2 Agonist and the FAAH Inhibitor JNJ Activated ERK but Suppressed p38

In comparison with the negative control samples, CB_2 agonism and FAAH inhibition suppressed p38 phosphorylation in both RASF and OASF, while the CB_1 agonist CP 49,497 and AEA activated p38 in RASF under hypoxia. Pre-incubation with the CB_1 inverse agonist

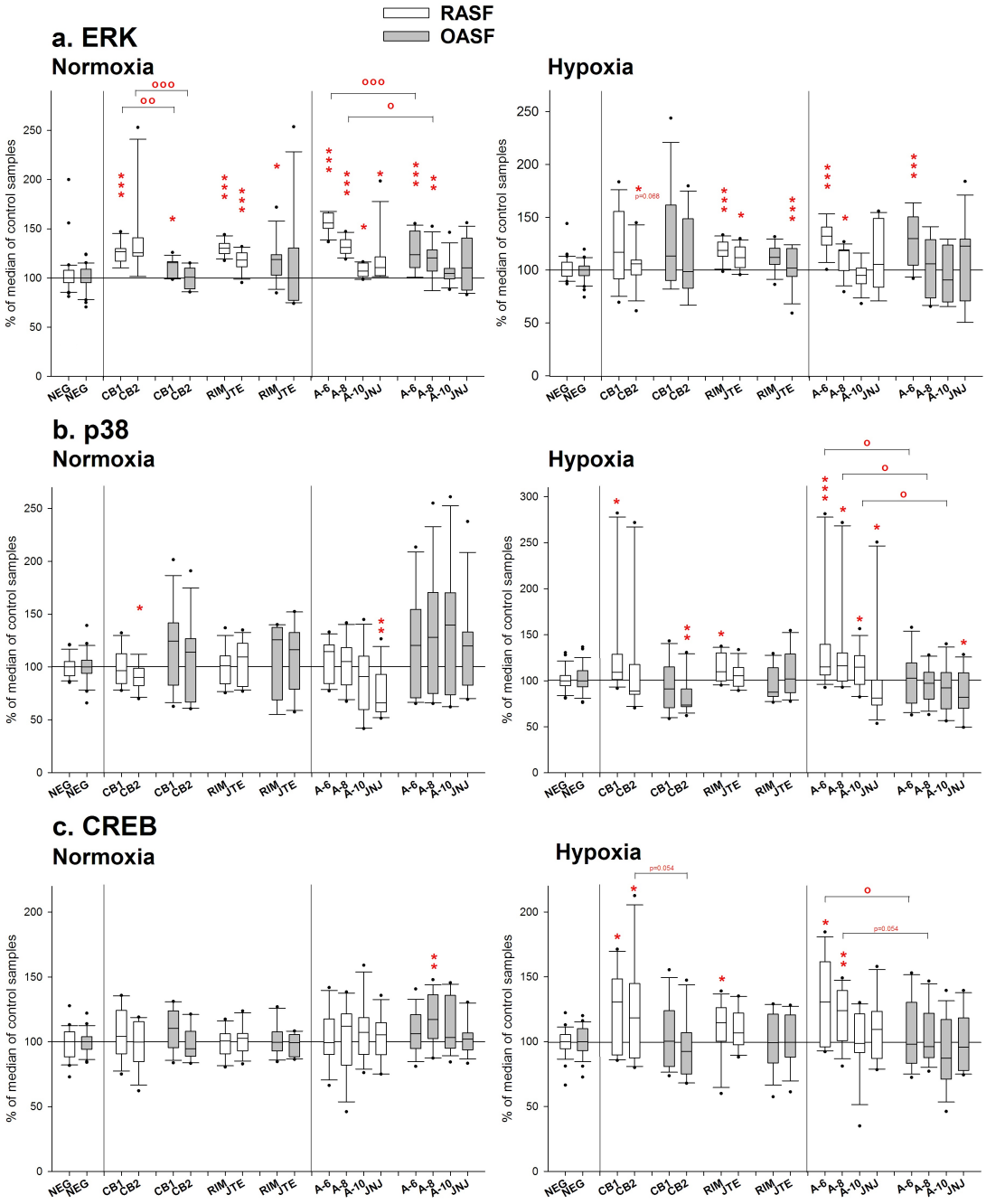

Figure 4.6: Rheumatoid arthritis synovial fibroblast (RASF, n=3) and osteoarthritis synovial fibroblasts (OASF, n=3) were incubated in normoxia or hypoxia with CB_1 agonist CP 47,497 (CB1, 1×10^{-6} M), CB_2 agonist GP1a (CB2, 1×10^{-7} M), inverse CB_1 agonist Rimonabant (RIM, 1×10^{-6} M), CB_2 inverse agonist JTE-907 (JTE, 1×10^{-7} M) as well as anandamide AEA (A-6, 1×10^{-6} M, A-8, 1×10^{-8} M or A-10,1×10^{-10} M) or fatty acid amide hydrolase JNJ (JNJ, 1×10^{-7} M). The level of phosphorylation of extracellular-signal regulated kinase (ERK), p38 MAPK (p38) and cAMP-responsive element binding protein (CREB) was measured with ELISA. Absorbance of the samples is given in percentage of median of the control samples on each plate. *= p<0.05, **= p<0.01, ***= p<0.001, vs. negative control sample (NEG). o= p<0.05, oo= p<0.01, ooo= p<0.001, vs. OASF.

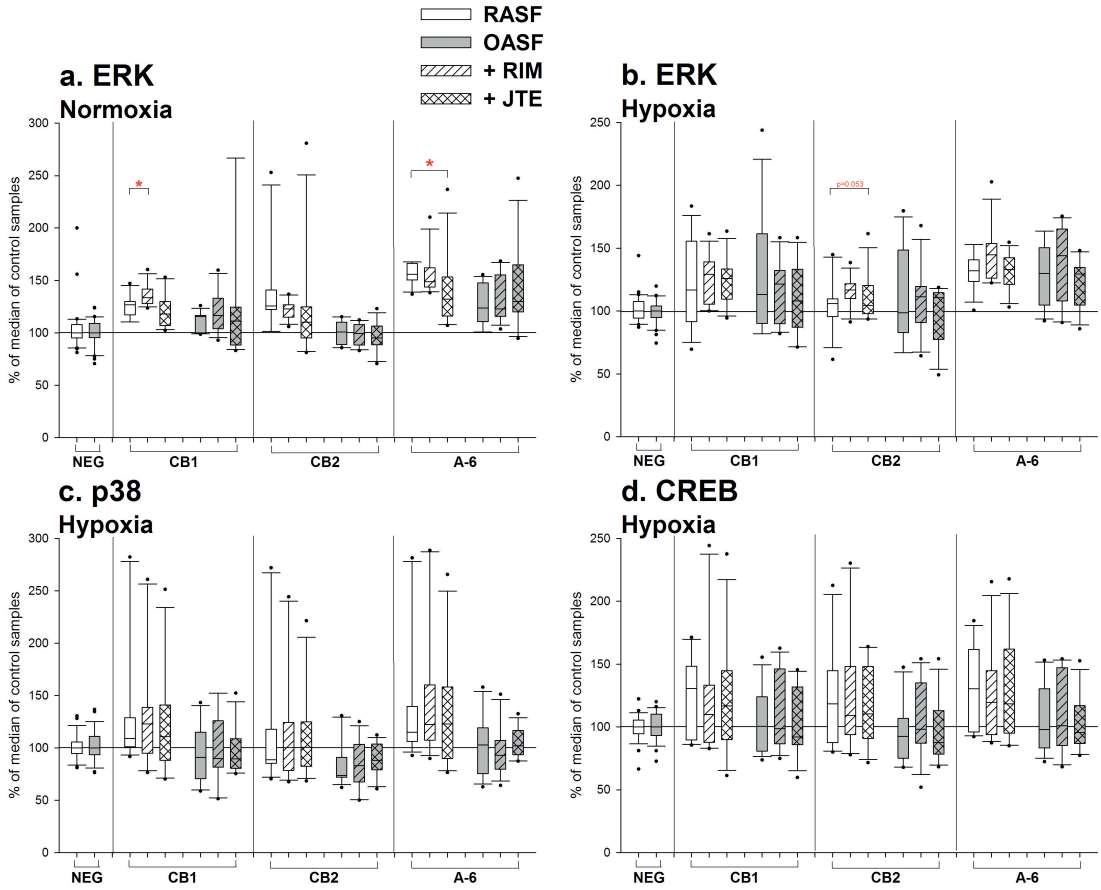

Figure 4.7: Rheumatoid arthritis synovial fibroblast (RASF, n=3) and osteoarthritis synovial fibroblasts (OASF, n=3) were first pretreated with the CB_1 inverse agonist Rimonabant (RIM, 1×10^{-6} M) or CB_2 inverse agonist JTE-907 (JTE, 1×10^{-7} M) and, subsequently, stimulated with the CB_1 agonist CP 47,497 (CB1, 1×10^{-6} M), CB_2 agonist GP1a (CB2, 1×10^{-7} M) or anandamide AEA (A-6, 1×10^{-6} M). The level of phosphorylation of extracellular-signal regulated kinase (ERK), p38 MAPK (p38) and cAMP-responsive element binding protein (CREB) was measured with ELISA. Absorbance of the samples is given in percentage of median of the control samples (NEG) on each plate. *= p<0.05, **= p<0.01, ***= p<0.001, vs. sample without pretreatment.

Rimonabant or CB_2 inverse agonist JTE did not alter these activation and suppression levels in p38 (Figure 4.7).

4.5.4 AEA and the CB_1 Agonist Showed Broadest Activation of MAPK

AEA (1×10^{-6} M) and the CB_1 agonist CP 47,497 demonstrated the strongest stimulation of MAPK in OASF and RASF under hypoxia and normoxia (Figure 4.6), compared to the negative control samples.

The CB_2 antagonist JTE-907 significantly reduced the activation of ERK by AEA in normoxia (Figure 4.7). After activation by the CB_1 agonist ACEA or CB_2 agonist GP1a, ERK phosphorylation peaked at 3 h in RASF and at 4 h in OASF (Figure 4.8).

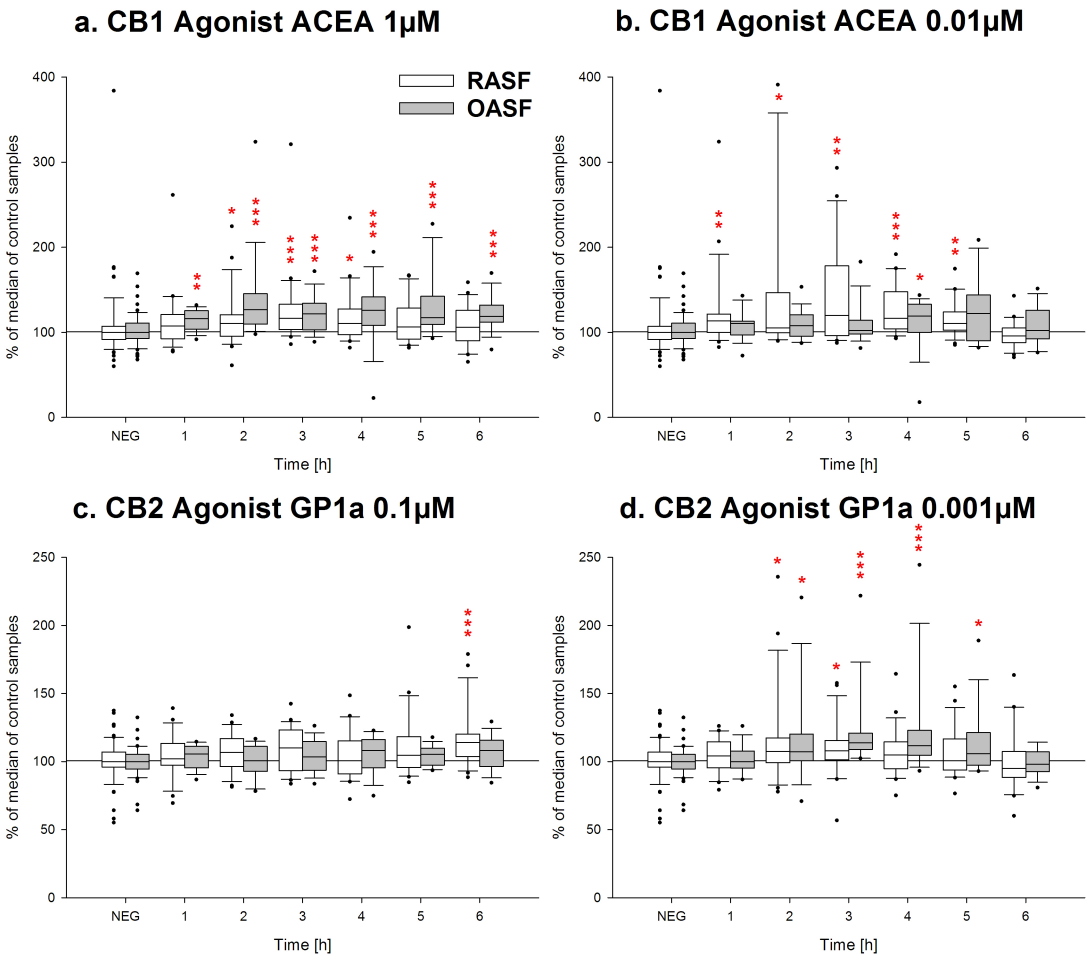

Figure 4.8: Rheumatoid arthritis synovial fibroblast (RASF, n=3) and osteoarthritis synovial fibroblasts (OASF, n=3) were stimulated with the CB_1 agonist ACEA (ACEA, 1×10^{-6} M) or CB_2 agonist GP1a (GP1a, 1×10^{-7} M) for 1 to 6 h. The level of phosphorylation of extracellular-signal regulated kinase ERK was measured with ELISA. Absorbance of the samples is given in percentage of median of the control samples on each plate. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, vs. negative control sample (NEG).

4.6 Conclusion: CB_1 Agonism is Mainly Responsible for Activation of MAPK and CREB in Synovial Fibroblasts

The CB_1 and CB_2 agonists as well as AEA phosphorylated MAPK both in RASF and OASF. Activation levels of ERK, p38 and CREB in RASF were significantly higher than in OASF (Figure 4.7).
Compared to the negative control samples, the CB_1 agonist CP 47,497, in particular, showed a specific activating effect on ERK and CREB but a suppressing effect on p38. The ERK phosphorylation by AEA was significantly lower after pretreatment with the CB_2 inverse agonist JTE than without pretreatment.
In experiments performed by Richardson et al., the CB_1 and CB_2 selective agonist Hu210 led to a $G_{\alpha i}$-protein-mediated phosphorylation of ERK1/2 and p38, which was blocked by a CB_1 antagonist but not a CB_2 antagonist (Richardson et al., 2008). Gui et al. demonstrated that Hu-210 decreased expression of IL-6, MMP-3 and MMP-13 (Gui et al., 2014).
The present studies were consistent with the fact, that CB_1 is the main cannabinoid receptor responsible for the phosphorylation of MAPK and CREB. For the first time, this study also demonstrated that AEA followed a similar pattern. However, the effects of AEA at high concentrations were not inhibited by CB_1 but CB_2 inverse agonists. This might be because AEA at high concentration may have off-target effects that lead to the activation of TRPV1. The precise involvement of both CB_1 and CB_2 receptors in anandamide activation remains to be further studied.

4.7 Consequences on Apoptosis and Cytokine Secretion

4.7.1 CB_1 Agonism Induced Apoptosis and Necrosis

In preliminary experiments, AEA and the CB_1 agonist CP 47,497 induced apoptosis and necrosis by an increase of cytochrome C production, Annexin-V and DNA presentation as well as LDH release. When compared to untreated control samples, incubation with CB_1

agonist CP 47,497, and not the CB_2 agonist GP1a, for 5 h induced an increase of cytochrome c expression in RASF (Figure 4.9a), and after 24 h, significantly increased the number of apoptotic and necrotic cells in OASF (Figure 4.11b). Likewise, 10 µM AEA significantly fostered necrosis in OASF after 24 h and led to a higher LDH release in both OASF and RASF than in untreated control samples (Figures 4.9 and 4.11b.).
Anandamide induced apoptosis on a wide range of cell types. Several mechanisms were proposed, such as MAPK p38 and JNK activation (Sarker et al., 2003; Hsu et al., 2007; Fonseca et al., 2013), direct involvement in the intrinsic and extrinsic apoptotic pathway (Gómez et al., 2014), ceramide production in decidual cells (Fonseca et al., 2013) or calcium influx through transient potential vanilloid Typ 1 receptors TRPV1 (Hu et al., 2012). Therefore, AEA might induce apoptosis also through a CB_1-mediated pathway. However, unlike the CB_1 agonist CP 47,497, AEA did not induce apoptosis but necrosis, probably by changing intracellular ceramids, calcium or lipid raft structures (Grimaldi et al., 2012).

4.7.2 AEA-Attenuated Expression of Key Cytokines After Stimulation by TNFα

In comparison to unpretreated samples, pre-incubation with AEA for 24 h tended to attenuate the increase in expression of the protein PAI-1 and the proinflammatory cytokines IL-6, MIF, CXCL10 and C5 after stimulation with TNFα for 5 min (Figure 4.12).
The plasminogen activator inhibitor-1 PAI-1 is known to be overexpressed in RASF and also responsible for the invasive and degradative character of RASF (Watanabe et al., 2002; Guiducci et al., 2005). The macrophage migration inhibitory factor (MIF-1) has a role in fibroblast proliferation (Lacey et al., 2003), angiogenesis (Kim et al., 2007) and MMP production (Pakozdi et al., 2006). The C-X-C motif chemokine 10 CXCL10 appears to have a complex role in the morphogenesis of RASF in the inflammatory setting (Laragione et al.,

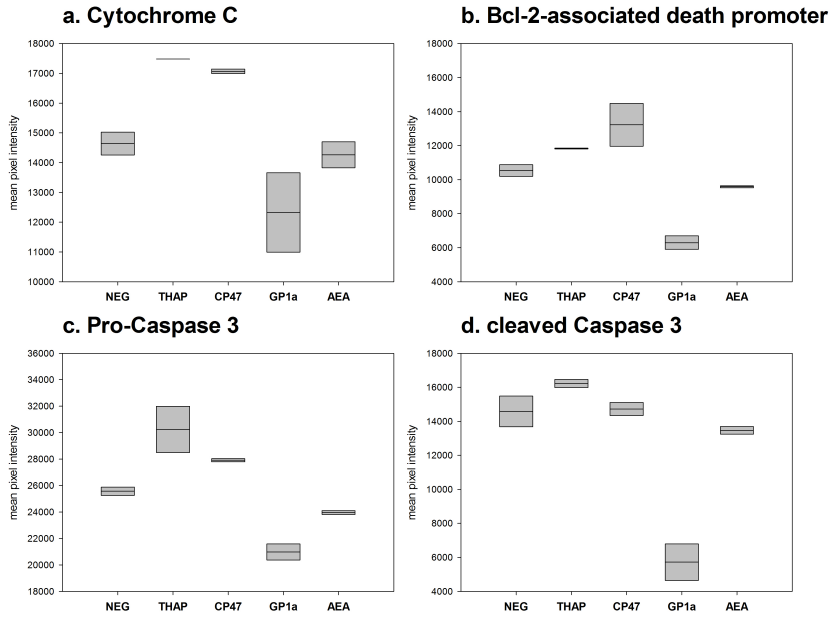

Figure 4.9: Rheumatoid arthritis synovial fibroblast (RASF, n=1) were incubated with the SERCA activator thapsigargin (THAP, 10 µM) as a positive control, the CB_1 agonist CP 47,497 (CP47, 1×10^{-6} M), CB_2 agonist GP1a (GP1a, 1×10^{-7} M) anandamide (AEA, 1×10^{-7} M) or not treated (NEG) in normoxia for 5 h. Samples are lysed and incubated on the Proteome Profiler™. Results are given in mean pixel intensities of the respective spots of the proteins on the slides after development of the chemiluminiscence for 5 min.

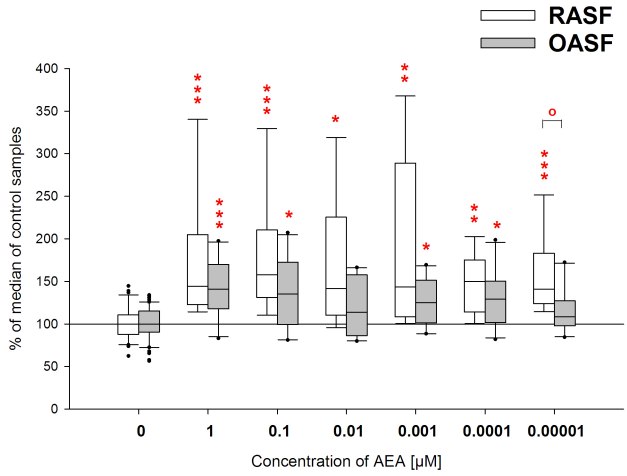

Figure 4.10: Rheumatoid arthritis synovial fibroblast (RASF, n=3) and osteoarthritis synovial fibroblasts (OASF, n=4) were incubated with anandamide (AEA) in different concentrations. After incubation for 24 h, concentration of lactate dehydrogenase in each well was measured with the Cytotoxicity Detection Kit. Absorbance of the samples is given in percentage of median of the control samples on each plate. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, vs. negative control sample (0).

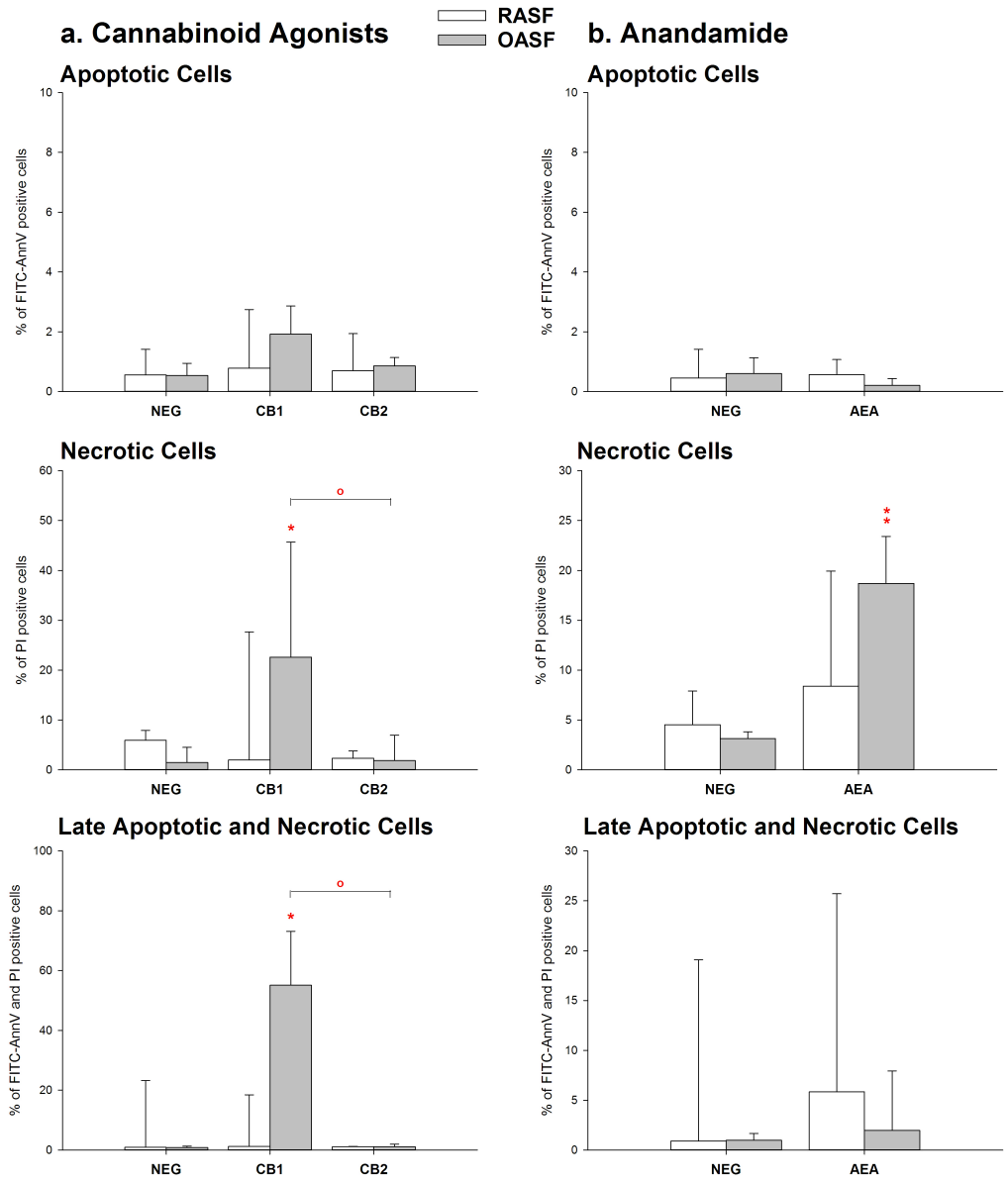

Figure 4.11: Rheumatoid arthritis synovial fibroblast (RASF, n=4) and osteoarthritis synovial fibroblasts (OASF, n=5) were incubated with the CB_1 agonist CP 47,497 (CB1, 1×10^{-6} M), CB_2 agonist GP1a (CB2, 1×10^{-7} M) or anandamide (AEA, 1×10^{-5} M). Using flow cytometry, Fluorescein Isothiocyanate (FITC)-Annexin V antibody detected apoptotic cells, propidiumiodine (PI) detected necrotic cells. *= $p<0.05$, **= $p<0.01$, vs. negative control sample (NEG). o= $p<0.05$, vs. CB_1/CB_2.

2011). The complement factor C5 and its fragment C5a is part of the pathogenic activation of the alternative pathway in the humoral immune defense, which mediates local arthritis (Arend et al., 2013). The MAPK system might be involved in the regulation of all these proteins. Therefore, changes in the TNFα-induced activation levels of the investigated MAPK might lead to the reduced expression of pro-inflammatory cytokines and reduced cartilage destruction.

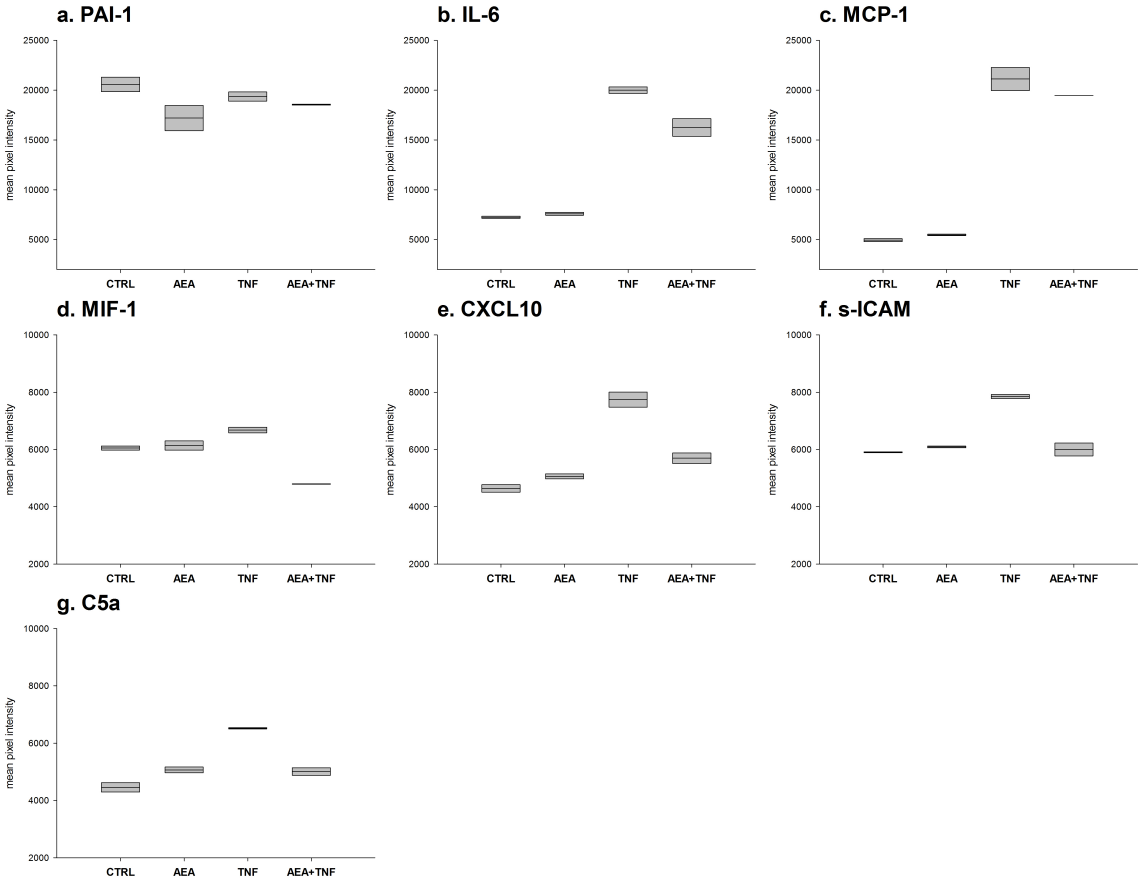

Figure 4.12: Rheumatoid arthritis synovial fibroblast (RASF, n=1) were incubated with anandamide AEA and TNFα for 24 h. In the supernatant, cytokines were detected using the Proteome Profiler™. Results are given in mean pixel intensities of the respective spots of the kinases on the slides after development of the chemiluminiscence for 5 min. PAI-1 denotes plasminogen activator inhibitor-1, IL-6 interleukin 6, MCP-1 monocyte chemoattractant protein 1, MIF-1 macrophage migration inhibitory factor 1, CXCL10 C-X-C Motif Ligand 10, s-ICAM soluble intercellular adhesion molecule and C5a complement factor fragment 5a.

4.8 Pathogenesis: The ECS and Immune System

The present study supports the idea of the CB_2 receptor being a counteracting component against the inflammatory process. This result might explain the increased expression of AEA in inflamed RA tissue (Richardson et al., 2008), whose synovial concentration of 1×10^{-9} M is in the range of its inhibitory effect. The actual effect, however, has still to be determined, since AEA has been shown to suppress secretion of pro-inflammatory cytokines by T-cells (Cencioni et al., 2010), but 2-arachidonylglycerol 2-AG, another endocannabinoid, has been associated with increased inflammation of the skin (Oka et al., 2006). In both cases, the CB_2 pathway is involved. It remains unkown whether AEA or 2-AG uses the same mechanism. Different affinities of AEA and 2-AG might explain this fact, since AEA only acts as a partial agonist, whereas 2-AG elicits full agonistic responses (Gonsiorek et al., 2000).
In addition to CB_1 and CB_2, other receptors and non-receptor pathways might influence the intracellular signaling and activation, e.g. the TRPV1 pathways (Alexander et al., 2011) or the balance of intracellular lipids (Fonseca et al., 2013).
Regarding the systemic role of the ECS, it is known to regulate energy homeostasis and lipid and glucose metabolism, e.g. in obesity, which is characterised by chronic inflammation and is associated with higher cyclooxygenase-2 and AEA levels (Hsieh et al., 2010; Silvestri et al., 2013). So far, only local effects of cannabinoids on the arthritic joint have been investigated. Still, its overall systemic influence and part in RA remain to be elucidated.

4.9 Therapy: CB_2 Agonism and the Anti-Inflammatory Treatment

Potent, peripherally acting CB_2 agonists could be appropropriate candidates for a supportive treatment with fewer adverse effects than traditional cannabinoids. They work in a host of cells, particularly on

the key triumvirate of synoviocytes, leukocytes and osteoclasts. The multicellular and pleiotropic effect of cannabinoid ligands, however, prevents a realistic prognosis of the treatment outcome. To further clarify local and systemic mechanisms and interactions, further studies still have to be conducted to verify the beneficial effects of cannabinoids.

For example, the cannabinoid Hu-308 is reported to attenuate the IL-1β induced secretion of IL-6, MMP-3 and MMP-13 in synovial fibroblasts (Gui et al., 2014). This suggests beneficial efficacy of cannabinoids to stop the inflammatory vicious cycle of fibroblasts and macrophages. In addition, secretion of degrading enzymes by fibroblasts and chrondrocytes is reduced after treatment with cannabinoids (Mbvundula et al., 2006). Also, the CB_2 agonist Hu-308 inhibits osteoclast formation (Ofek et al., 2006), suggesting that the cannabinoid treatment might also be beneficial for the treatment of bone loss in RA.

Therapeutic interventions targeting the endocannabinoid system still need to be validated for efficacy and efficiency in randomized controlled trials. For the time being, it is inappropriate to exaggerate potential advantages and underestimate the disadvantages of selective cannabinoids, as high expectations about the anti-atheroslerotic effect of rimonabant were disappointed in the STRADIVARIUS trial (Nissen et al., 2008). While inducing more psychiatric side effects, the CB_1 antagonist rimonabant did not have a favourable influence on the disease progression of atherosclerosis in patients with abdominal obesity (Nissen et al., 2008).

Summary and Outlook

Fibroblast-like synoviocytes are the key aggressive players in rheumatoid arthritis and largely responsible for inflammation sustenance and joint destruction. Mitigation of the aggressive behaviour and elimination of the apoptotic resistance is crucial, yet still has not been sufficiently addressed in current therapy regimes, even after the introduction of biological therapies. The recent discovery of the cannabinoid system in the joint might address this challenge.

New innovative CB_2-selective agonists with less psychoactive effects are increasingly developed for anti-inflammatory treatment on a wide range of cell types (Fukuda et al., 2014; Dunn et al., 2014). In addition, regulation of the endocannabinoid levels by using FAAH inhibitors might also be an effective strategy. FAAH inhibitors have shown to reduce arthritis (Kinsey et al., 2011) and to increase glucocorticoid effects on synovial fibroblasts (Lowin et al., 2012). Possible treatment options might also include both strategies, since raising the endogenous level of AEA might boost the therapeutic power of the cannabinoid agonists.

Yet, its pleiotropic effects on various types of cells involved in RA requires additional investigations *in vivo* to discern its usefulness in RA therapy. The complex nature of the endocannabinoid system could be its great strength or stumbling block.

Abbreviations

2-AG	2-Arachidonylglycerol
7-AAD	7-Aminoactinomycin D
ACPA	Anti-citrullinated Peptide Antibodies
ACR	American College of Rheumatology
AEA	Anandamide
AP-1	Activator Protein 1
BCL-2	B-Cell Lymphoma Protein 2
C5	Complement Factor Fragment 5
Ca2+	Ionized Calcium
cAMP	Cyclic Adenosine Monophosphate
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
CCL2	C-C Motif Ligand 2
CCL5	C-C Motif Ligand 5
CD95	Cluster of Differentiation 95
COX-2	Cyclooxyenase 2
CREB	cAMP-Responsive Element Binding Protein
CXCL10	C-X-C Motif Ligand 10
CXCL12	C-X-C Motif Ligand 12
CXCL13	C-X-C Motif Ligand 13
CXCR4	C-X-C Motif Receptor 4
CXCR5	C-X-C Motif Receptor 5
DMARD	Disease-Modifying Anti-Rheumatic Drug
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic Acid
ECS	Endocannabinoid System
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular-Signal Regulated Kinase
FAAH	Fatty Amide Acid Hydrolase
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GCPR	G protein-Coupled Receptor Family
GPR55	G Protein-Coupled Receptor 55

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HLA-DR	Human Leukocyte Antigen DR chain
ICAM	Intercellular Adhesion Molecule
IL-1	Interleukin 1
IL-6	Interleukin 6
JNK	c-Jun N-Terminal Kinase
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCL-2	Myleoid Cell Leukemia Protein 2
MCP-1	Monocyte Chemoattractant Protein 1
MIF-1	Macrophage Migration Inhibitory Factor 1
miRNA	Micro Ribonucleic Acid
MMP	Matrix Metalloproteinase
MOMP	Mitochondrial Outer Membrane Permeabilization
MSK	Mitogen- and Stress-Activated Protein Kinase
NFκB	Nuclear Factor κ-Light Chain Enhancer of Activated B-Cells
NSAID	Non-Steroidal Anti-Inflammatory Drug
OA	Osteoarthritis
OASF	Osteoarthritis Synovial Fibroblasts
p38	Protein 38
P53	Tumor Proteins 53
PADI4	Peptidyl Arginine Deiminase Type 4
PAI-1	Plasminogen Activator Inhibitor 1
PBS	Phosphate-Buffered Saline
PI	Propidium Iodide
PTPN22	Protein Tyrosine Phosphatase, Non-receptor type 22
RA	Rheumatoid Arthritis
RASF	Rheumatoid Arthritis Synovial Fibroblasts
RF	Rheumatoid Factor
RhoA	Ras Homolog Gene Family Member A
RPMI	Roswell Park Memorial Institute
RSK	90 kDa Ribosomal S6 Kinase
SF	Synovial Fibroblasts
STAT	Signal Transducer and Activator of Transcription protein
TGF	Transforming Growth Factor
TH-17	Helper T-Cell Subtype 17
TMB	3,3',5,5'-Tetramethylbenzidin
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRPV	Transient Receptor Potential Channels of the Vanilloid Family
VCAM	Vascular Cell Adhesion Molecule

List of Figures

List of Tables

Bibliography

Agarwal, Nitin et al. (2007): 'Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors'. *Nature neuroscience*, vol. 10(7): pp. 870–879.

Alexander, S. P.H., A. Mathie, and J. A. Peters (2011): 'Special Issue: Guide to Receptors and Channels, 5th Edition Abstracts'. *British Journal of Pharmacology*, vol. 164: S1–+.

Almeida, Denise E. de, Song Ling, Xiujun Pi, Anne M. Hartmann-Scruggs, Paul Pumpens, and Joseph Holoshitz (2010): 'Immune dysregulation by the rheumatoid arthritis shared epitope'. *Journal of Immunology (Baltimore, Md. : 1950)*, vol. 185(3): pp. 1927–1934.

Altarejos, Judith Y. and Marc Montminy (2011): 'CREB and the CRTC co-activators: sensors for hormonal and metabolic signals'. *Nature Reviews. Molecular Cell Biology*, vol. 12(3): pp. 141–151.

Amft, N., S. J. Curnow, D. Scheel-Toellner, A. Devadas, J. Oates, J. Crocker, J. Hamburger, J. Ainsworth, J. Mathews, M. Salmon, S. J. Bowman, and C. D. Buckley (2001): 'Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjögren's syndrome'. *Arthritis and Rheumatism*, vol. 44(11): pp. 2633–2641.

Arend, William P., Gaurav Mehta, Alexandra H. Antonioli, Minoru Takahashi, Kazue Takahashi, Gregory L. Stahl, V. Michael Holers, and Nirmal K. Banda (2013): 'Roles of adipocytes and fibroblasts in activation of the alternative pathway of complement in inflammatory arthritis in mice'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 190(12): pp. 6423–6433.

Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, and H. S. Luthra (1988): 'The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis'. *Arthritis and rheumatism*, vol. 31(3): pp. 315–324.

Barchowsky, A., D. Frleta, and M. P. Vincenti (2000): 'Integration of the NF-kappaB and mitogen-activated protein kinase/AP-1 pathways at the collagenase-1 promoter: divergence of IL-1 and TNF-dependent signal transduction in rabbit primary synovial fibroblasts'. *Cytokine*, vol. 12(10): pp. 1469–1479.

Bartok, Beatrix and G. S. Firestein (2010): 'Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis'. *Immunological Reviews*, vol. 233(1): pp. 233–255.

Begovich, Ann B. et al. (2004): 'A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis'. *American Journal of Human Genetics*, vol. 75(2): pp. 330–337.

Bifulco, Maurizio, Chiara Laezza, Patrizia Gazzerro, and Francesca Pentimalli (2007): 'Endocannabinoids as emerging suppressors of angiogenesis and tumor invasion (review)'. *Oncology reports*, vol. 17(4): pp. 813–816.

Bisogno, T. and V. Di Marzo (2007): 'Short- and long-term plasticity of the endocannabinoid system in neuropsychiatric and neurological disorders'. *Pharmacological research*, vol. 56(5): pp. 428–442.

Bottini, Nunzio and G. S. Firestein (2013): 'Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors'. *Nature reviews. Rheumatology*, vol. 9(1): pp. 24–33.

Brauchle, M., D. Glück, F. Di Padova, J. Han, and H. Gram (2000): 'Independent role of p38 and ERK1/2 mitogen-activated kinases in the upregulation of matrix metalloproteinase-1'. *Experimental cell research*, vol. 258(1): pp. 135–144.

Buckley, C. D., D. Pilling, J. M. Lord, A. N. Akbar, D. Scheel-Toellner, and M. Salmon (2001): 'Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation'. *Trends in immunology*, vol. 22(4): pp. 199–204.

Burger, J. A., N. J. Zvaifler, N. Tsukada, G. S. Firestein, and T. J. Kipps (2001): 'Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism'. *The Journal of clinical investigation*, vol. 107(3): pp. 305–315.

Calmon-Hamaty, Flavia, Bernard Combe, Michael Hahne, and Jacques Morel (2011): 'Lymphotoxin a stimulates proliferation and pro-inflammatory cytokine secretion of rheumatoid arthritis synovial fibroblasts'. *Cytokine*, vol. 53(2): pp. 207–214.

Cantaert, Tineke, Sophie Brouard, Rogier M. Thurlings, Annaick Pallier, Gabriela Franco Salinas, Christophe Braud, Paul L. Klarenbeek, Niek de Vries, Yiping Zhang, Jean-Paul Soulillou, Paul P. Tak, and Dominique Baeten (2009): 'Alterations of the synovial T cell repertoire in anti-citrullinated protein antibody-positive rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 60(7): pp. 1944–1956.

Capellino, Silvia, Marco Cosentino, Christine Wolff, Martin Schmidt, Joachim Grifka, and Rainer H. Straub (2010): 'Catecholamine-producing cells in the synovial tissue during arthritis: modulation of sympathetic neurotransmitters as new therapeutic target'. *Annals of the rheumatic diseases*, vol. 69(10): pp. 1853–1860.

Cell Signaling Technology, Inc (2010): *MAPK*. Ed. by Cell Signaling Technology, Inc.

Cencioni, Maria Teresa, Valerio Chiurchiù, Giuseppina Catanzaro, Giovanna Borsellino, Giorgio Bernardi, Luca Battistini, and Mauro Maccarrone (2010): 'Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors'. *PloS one*, vol. 5(1): e8688.

Chang, Sook Kyung, Erika H. Noss, Mei Chen, Zhizhan Gu, Kirk Townsend, Rosa Grenha, Luis Leon, Soo Young Lee, David M. Lee, and Michael B. Brenner (2011): 'Cadherin-11 regulates fibroblast inflammation'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108(20): pp. 8402–8407.

Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman (1993): 'Phosphorylated CREB binds specifically to the nuclear protein CBP'. *Nature*, vol. 365(6449): pp. 855–859.

Cornish, Ann L., Ian K. Campbell, Brent S. McKenzie, Simon Chatfield, and Ian P. Wicks (2009): 'G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis'. *Nature reviews. Rheumatology*, vol. 5(10): pp. 554–559.

Damjanov, Nemanja, Robert S. Kauffman, and George T. Spencer-Green (2009): 'Efficacy, pharmacodynamics, and safety of VX-702, a novel p38 MAPK inhibitor, in rheumatoid arthritis: results of two randomized, double-blind, placebo-controlled clinical studies'. *Arthritis and Rheumatism*, vol. 60(5): pp. 1232–1241.

D'argenio, Giuseppe, Marta Valenti, Giuseppe Scaglione, Vittorio Cosenza, Italo Sorrentini, and Vincenzo Di Marzo (2006): 'Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation'. *FASEB*

journal : official publication of the Federation of American Societies for Experimental Biology, vol. 20(3): pp. 568–570.

Dash, P. K., K. A. Karl, M. A. Colicos, R. Prywes, and E. R. Kandel (1991): 'cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88(11): pp. 5061–5065.

Davis, John M., Véronique L. Roger, Cynthia S. Crowson, Hilal Maradit Kremers, Terry M. Therneau, and Sherine E. Gabriel (2008): 'The presentation and outcome of heart failure in patients with rheumatoid arthritis differs from that in the general population'. *Arthritis and Rheumatism*, vol. 58(9): pp. 2603–2611.

Deak, M., A. D. Clifton, L. M. Lucocq, and D. R. Alessi (1998): 'Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB'. *The EMBO journal*, vol. 17(15): pp. 4426–4441.

Devane, W. A., L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam (1992): 'Isolation and structure of a brain constituent that binds to the cannabinoid receptor'. *Science (New York, N.Y.)* Vol. 258(5090): pp. 1946–1949.

Di Marzo, Vincenzo and A. A. Izzo (2006): 'Endocannabinoid overactivity and intestinal inflammation'. *Gut*, vol. 55(10): pp. 1373–1376.

Di Marzo, Vincenzo and Stefania Petrosino (2007): 'Endocannabinoids and the regulation of their levels in health and disease'. *Current opinion in lipidology*, vol. 18(2): pp. 129–140.

Dou, Yannong, Bei Tong, Zhifeng Wei, Ying Li, Yufeng Xia, and Yue Dai (2013): 'Scopoletin suppresses IL-6 production from fibroblast-like synoviocytes of adjuvant arthritis rats induced by IL-1b stimulation'. *International immunopharmacology*, vol. 17(4): pp. 1037–1043.

Dunn, S. L., J. M. Wilkinson, A. Crawford, Le Maitre, C L, and Bunning, R A D (2014): 'Cannabinoid WIN-55,212-2 mesylate inhibits interleukin-1b induced matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression in human chondrocytes'. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*, vol. 22(1): pp. 133–144.

Eyles, Jo L., Andrew W. Roberts, Donald Metcalf, and Ian P. Wicks (2006): 'Granulocyte colony-stimulating factor and neutrophils–forgotten mediators of inflammatory disease'. *Nature clinical practice. Rheumatology*, vol. 2(9): pp. 500–510.

Firestein, G. S. (2003): 'Evolving concepts of rheumatoid arthritis'. *Nature*, vol. 423(6937): pp. 356–361.

Firestein, G. S., Ralph C. Budd, Sherine E. Gabriel, Iain B. McInnes, and James R. O'Dell (2012): *Kelley's Textbook of Rheumatology: Expert Consult Premium Edition: Enhanced Online Features*. 9th ed. s.l: Elsevier Health Care - Major Reference Works.

Firestein, G. S., F. Echeverri, M. Yeo, N. J. Zvaifler, and D. R. Green (1997): 'Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94(20): pp. 10895–10900.

Fonseca, B. M., G. Correia-da-Silva, and N. A. Teixeira (2013): 'The endocannabinoid anandamide induces apoptosis of rat decidual cells through a mechanism involving ceramide synthesis and p38 MAPK activation'. *Apoptosis : an international journal on programmed cell death*, vol. 18(12): pp. 1526–1535.

Fukuda, Shin, Hitoshi Kohsaka, Aiko Takayasu, Waka Yokoyama, Chie Miyabe, Yoshishige Miyabe, Masayoshi Harigai, Nobuyuki Miyasaka, and Toshihiro Nanki (2014): 'Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis'. *BMC musculoskeletal disorders*, vol. 15: p. 275.

Furneri, Gianluca, Lorenzo G. Mantovani, Andrea Belisari, Marta Mosca, Marco Cristiani, Stefania Bellelli, Paolo A. Cortesi, and Giuseppe Turchetti (2012): 'Sys-

tematic literature review on economic implications and pharmacoeconomic issues of rheumatoid arthritis'. *Clinical and experimental rheumatology*, vol. 30(4 Suppl 73): S72–84.

Gabriel, S. E. and K. Michaud (2009): 'Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases'. *Arthritis research & therapy*, vol. 11(3):

Genovese, Mark C., Stanley B. Cohen, David Wofsy, Michael E. Weinblatt, G. S. Firestein, Ernest Brahn, Vibeke Strand, Daniel G. Baker, and Sandra E. Tong (2011): 'A 24-week, randomized, double-blind, placebo-controlled, parallel group study of the efficacy of oral SCIO-469, a p38 mitogen-activated protein kinase inhibitor, in patients with active rheumatoid arthritis'. *The Journal of rheumatology*, vol. 38(5): pp. 846–854.

Gérard, C., C. Mollereau, G. Vassart, and M. Parmentier (1990): 'Nucleotide sequence of a human cannabinoid receptor cDNA'. *Nucleic acids research*, vol. 18(23): p. 7142.

Gómez, Rodolfo, Javier Conde, Morena Scotece, Verónica López, Francisca Lago, Gómez Reino, Juan Jesús, and Oreste Gualillo (2014): 'Endogenous cannabinoid anandamide impairs cell growth and induces apoptosis in chondrocytes'. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, vol. 32(9): pp. 1137–1146.

Gonsiorek, W., C. Lunn, X. Fan, S. Narula, D. Lundell, and R. W. Hipkin (2000): 'Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide'. *Molecular pharmacology*, vol. 57(5): pp. 1045–1050.

Gonzalez, G. A. and M. R. Montminy (1989): 'Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133'. *Cell*, vol. 59(4): pp. 675–680.

Görtz, Birgit, Silvia Hayer, Birgit Tuerck, Jochen Zwerina, Josef S. Smolen, and Georg Schett (2005): 'Tumour necrosis factor activates the mitogen-activated protein kinases p38alpha and ERK in the synovial membrane in vivo'. *Arthritis research & therapy*, vol. 7(5): R1140–7.

Grabiec, Aleksander M. and Kris A. Reedquist (2013): 'The ascent of acetylation in the epigenetics of rheumatoid arthritis'. *Nature reviews. Rheumatology*, vol. 9(5): pp. 311–318.

Gregersen, P. K., J. Silver, and R. J. Winchester (1987): 'The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 30(11): pp. 1205–1213.

Grimaldi, Claudia and Anna Capasso (2012): 'Role of lipid rafts/caveolae in the anticancer effect of endocannabinoids'. *Mini reviews in medicinal chemistry*, vol. 12(11): pp. 1119–1126.

Gui, Huan, Xia Liu, Zhi-Wei Wang, Dong-Yi He, Ding-Feng Su, and Sheng-Ming Dai (2014): 'Expression of cannabinoid receptor 2 and its inhibitory effects on synovial fibroblasts in rheumatoid arthritis'. *Rheumatology (Oxford, England)*, vol.

Guiducci, S., A. Del Rosso, M. Cinelli, F. Margheri, S. D'Alessio, G. Fibbi, M. Matucci Cerinic, and M. Del Rosso (2005): 'Rheumatoid synovial fibroblasts constitutively express the fibrinolytic pattern of invasive tumor-like cells'. *Clinical and experimental rheumatology*, vol. 23(3): pp. 364–372.

Guzmán, Manuel (2003): 'Cannabinoids: potential anticancer agents'. *Nature reviews. Cancer*, vol. 3(10): pp. 745–755.

Hagel, Sofia, Ingemar F. Petersson, Ann Bremander, Elisabet Lindqvist, Charlotte Bergknut, and Martin Englund (2013): 'Trends in the first decade of 21st century healthcare utilisation in a rheumatoid arthritis cohort compared with the general population'. *Annals of the rheumatic diseases*, vol. 72(7): pp. 1212–1216.

Han, Z. N., D. L. Boyle, K. R. Aupperle, B. Bennett, Am Manning, and G. S. Firestein (1999): 'Jun N-terminal kinase in rheumatoid arthritis'. *Journal of pharmacology and experimental therapeutics*, vol. 291(1): pp. 124–130.

Han, Z., D. L. Boyle, L. Chang, B. Bennett, M. Karin, L. Yang, A. M. Manning, and G. S. Firestein (2001): 'c-Jun N-terminal kinase is required for metalloproteinase expression

and joint destruction in inflammatory arthritis'. *The Journal of clinical investigation*, vol. 108(1): pp. 73–81.

Haringman, J. J., D. M. Gerlag, A. H. Zwinderman, T. J. M. Smeets, M. C. Kraan, D. Baeten, I. B. McInnes, B. Bresnihan, and P. P. Tak (2005): 'Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis'. *Annals of the rheumatic diseases*, vol. 64(6): pp. 834–838.

Härle, Peter, Daniel Mobius, Carr, Daniel J J, Jurgen Scholmerich, and Rainer H. Straub (2005): 'An opposing time-dependent immune-modulating effect of the sympathetic nervous system conferred by altering the cytokine profile in the local lymph nodes and spleen of mice with type II collagen-induced arthritis'. *Arthritis and rheumatism*, vol. 52(4): pp. 1305–1313.

Hohmann, Andrea G. and Richard L. Suplita (2006): 'Endocannabinoid mechanisms of pain modulation'. *The AAPS journal*, vol. 8(4): E693–708.

Hot, Arnaud and Pierre Miossec (2011): 'Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes'. *Annals of the rheumatic diseases*, vol. 70(5): pp. 727–732.

Hsieh, P-S, K-C Lu, C-F Chiang, and C-H Chen (2010): 'Suppressive effect of COX2 inhibitor on the progression of adipose inflammation in high-fat-induced obese rats'. *European journal of clinical investigation*, vol. 40(2): pp. 164–171.

Hsu, Shu-Shong, Chun-Jen Huang, He-Hsiung Cheng, Chiang-Ting Chou, Hsiao-Ying Lee, Jue-Long Wang, I-Shu Chen, Shiuh-Inn Liu, Yih-Chau Lu, Hong-Tai Chang, Jong-Khing Huang, Jin-Shyr Chen, and Chung-Ren Jan (2007): 'Anandamide-induced Ca2+ elevation leading to p38 MAPK phosphorylation and subsequent cell death via apoptosis in human osteosarcoma cells'. *Toxicology*, vol. 231(1): pp. 21–29.

Hu, Fen, Shuang Yang, Dan Zhao, Shuyan Zhu, Yuxiang Wang, and Junying Li (2012): 'Moderate extracellular acidification inhibits capsaicin-induced cell death through regulating calcium mobilization, NF-kB translocation and ROS production in synoviocytes'. *Biochemical and biophysical research communications*, vol. 424(1): pp. 196–200.

Huscher, D., S. Merkesdal, K. Thiele, H. Zeidler, M. Schneider, and A. Zink (2006): 'Cost of illness in rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis and systemic lupus erythematosus in Germany'. *Annals of the rheumatic diseases*, vol. 65(9): pp. 1175–1183.

Huscher, D., T. Mittendorf, U. von Hinüber, I. Kötter, G. Hoese, A. Pfäfflin, S. Bischoff, and A. Zink (2014): 'Evolution of cost structures in rheumatoid arthritis over the past decade'. *Annals of the rheumatic diseases*, vol.

Imamura, F., H. Aono, T. Hasunuma, T. Sumida, H. Tateishi, S. Maruo, and K. Nishioka (1998): 'Monoclonal expansion of synoviocytes in rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 41(11): pp. 1979–1986.

Impey, Soren, Sean R. McCorkle, Hyunjoo Cha-Molstad, Jami M. Dwyer, Gregory S. Yochum, Jeremy M. Boss, Shannon McWeeney, John J. Dunn, Gail Mandel, and Richard H. Goodman (2004): 'Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions'. *Cell*, vol. 119(7): pp. 1041–1054.

Inoue, Tomoyuki, David L. Boyle, Maripat Corr, Deepa Hammaker, Roger J. Davis, Richard A. Flavell, and G. S. Firestein (2006): 'Mitogen-activated protein kinase kinase 3 is a pivotal pathway regulating p38 activation in inflammatory arthritis'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103(14): pp. 5484–5489.

Inoue, Tomoyuki, Deepa Hammaker, David L. Boyle, and G. S. Firestein (2005): 'Regulation of p38 MAPK by MAPK kinases 3 and 6 in fibroblast-like synoviocytes'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 174(7): pp. 4301–4306.

Ishizu, Akihiro, Asami Abe, Yukiko Miyatake, Tomohisa Baba, Chihiro Iinuma, Utano Tomaru, and Takashi Yoshiki (2010): 'Cyclic AMP response element-binding protein is implicated in IL-6 production from arthritic synovial cells'. *Modern rheumatology / the Japan Rheumatism Association*, vol. 20(2): pp. 134–138.

Iversen, L. (1999): 'High times for cannabis research'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96(10): pp. 5338–5339.

Jhaveri, M. D., D. Richardson, and V. Chapman (2007): 'Endocannabinoid metabolism and uptake: novel targets for neuropathic and inflammatory pain'. *British journal of pharmacology*, vol. 152(5): pp. 624–632.

Johnson, G. L. and Razvan Lapadat (2002): 'Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases'. *Science (New York, N.Y.)* Vol. 298(5600): pp. 1911–1912.

Källberg, Henrik, Bo Ding, Leonid Padyukov, Camilla Bengtsson, Johan Rönnelid, Lars Klareskog, and Lars Alfredsson (2011): 'Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke'. *Annals of the rheumatic diseases*, vol. 70(3): pp. 508–511.

Kamphuis, Sylvia, Wietse Kuis, Wilco de Jager, Gijs Teklenburg, Margherita Massa, Grace Gordon, Marjolein Boerhof, Ger T. Rijkers, Cuno S. Uiterwaal, Henny G. Otten, Alessandro Sette, Salvatore Albani, and Berent J. Prakken (2005): 'Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis'. *Lancet*, vol. 366(9479): pp. 50–56.

Karbarz, Mark J., Lin Luo, Leon Chang, Chui-Se Tham, James A. Palmer, Sandy J. Wilson, Michelle L. Wennerholm, Sean M. Brown, Brian P. Scott, Richard L. Apodaca, John M. Keith, Jiejun Wu, James Guy Breitenbucher, Sandra R. Chaplan, and Michael Webb (2009): 'Biochemical and biological properties of 4-(3-phenyl-[1,2,4] thiadiazol-5-yl)-piperazine-1-carboxylic acid phenylamide, a mechanism-based inhibitor of fatty acid amide hydrolase'. *Anesthesia and analgesia*, vol. 108(1): pp. 316–329.

Karsak, Meliha et al. (2007): 'Attenuation of allergic contact dermatitis through the endocannabinoid system'. *Science (New York, N.Y.)* Vol. 316(5830): pp. 1494–1497.

Kim, Hae-Rim, Mi-Kyung Park, Mi-La Cho, Chong-Hyeon Yoon, Sang-Heon Lee, Sung-Hwan Park, Lin Leng, Richard Bucala, Insoo Kang, Jongseon Choe, and Ho-Youn Kim (2007): 'Macrophage migration inhibitory factor upregulates angiogenic factors and correlates with clinical measures in rheumatoid arthritis'. *The Journal of rheumatology*, vol. 34(5): pp. 927–936.

Kinsey, Steven G., Pattipati S. Naidu, Benjamin F. Cravatt, David T. Dudley, and Aron H. Lichtman (2011): 'Fatty acid amide hydrolase blockade attenuates the development of collagen-induced arthritis and related thermal hyperalgesia in mice'. *Pharmacology, biochemistry, and behavior*, vol. 99(4): pp. 718–725.

Kishimoto, Tadamitsu (1989): 'The biology of interleukin-6'. *Blood*, vol. 74(1): pp. 1–10.

Klareskog, Lars, Patrik Stolt, Karin Lundberg, Henrik Källberg, Camilla Bengtsson, Johan Grunewald, Johan Rönnelid, Helena Erlandsson Harris, Ann-Kristin Ulfgren, Solbritt Rantapää-Dahlqvist, Anders Eklund, Leonid Padyukov, and Lars Alfredsson (2006): 'A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination'. *Arthritis and Rheumatism*, vol. 54(1): pp. 38–46.

Kleyer, Arnd and Georg Schett (2014): 'Arthritis and bone loss: a hen and egg story'. *Current opinion in rheumatology*, vol. 26(1): pp. 80–84.

Lacey, Derek, Annaleise Sampey, Robert Mitchell, Richard Bucala, Lanie Santos, Michelle Leech, and Eric Morand (2003): 'Control of fibroblast-like synoviocyte proliferation by macrophage migration inhibitory factor'. *Arthritis and Rheumatism*, vol. 48(1): pp. 103–109.

Lally, Frank, Emily Smith, Andrew Filer, Michael A. Stone, John S. Shaw, Gerard B. Nash, Christopher D. Buckley, and G. Ed Rainger (2005): 'A novel mechanism of neutrophil recruitment in a coculture model of the rheumatoid synovium'. *Arthritis and Rheumatism*, vol. 52(11): pp. 3460–3469.

Laragione, Teresina, Max Brenner, Barbara Sherry, and Pércio S. Gulko (2011): 'CXCL10 and its receptor CXCR3 regulate synovial fibroblast invasion in rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 63(11): pp. 3274–3283.

Lee, David M., Hans P. Kiener, Sandeep K. Agarwal, Erika H. Noss, Gerald F. M. Watts, Osamu Chisaka, Masatoshi Takeichi, and Michael B. Brenner (2007): 'Cadherin-11 in synovial lining formation and pathology in arthritis'. *Science (New York, N.Y.)* Vol. 315(5814): pp. 1006–1010.

Lefèvre, Stephanie et al. (2009): 'Synovial fibroblasts spread rheumatoid arthritis to unaffected joints'. *Nature medicine*, vol. 15(12): pp. 1414–1420.

Li, W. Q., F. Dehnade, and M. Zafarullah (2001): 'Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 166(5): pp. 3491–3498.

Link, Tiffany M., Una Park, Becky M. Vonakis, Daniel M. Raben, Mark J. Soloski, and Michael J. Caterina (2010): 'TRPV2 has a pivotal role in macrophage particle binding and phagocytosis'. *Nature immunology*, vol. 11(3): pp. 232–239.

Liu, Hongtao, Polikseni Eksarko, Vladislav Temkin, G. Kenneth Haines, Harris Perlman, Alisa E. Koch, Bayar Thimmapaya, and Richard M. Pope (2005): 'Mcl-1 is essential for the survival of synovial fibroblasts in rheumatoid arthritis'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 175(12): pp. 8337–8345.

Lowin, Torsten, Wentao Zhu, Katja Dettmer-Wilde, and Rainer H. Straub (2012): 'Cortisol-mediated adhesion of synovial fibroblasts is dependent on the degradation of anandamide and activation of the endocannabinoid system'. *Arthritis and Rheumatism*, vol. 64(12): pp. 3867–3876.

Lunn, C. A., E-P Reich, J. S. Fine, B. Lavey, J. A. Kozlowski, R. W. Hipkin, D. J. Lundell, and L. Bober (2008): 'Biology and therapeutic potential of cannabinoid CB2 receptor inverse agonists'. *British journal of pharmacology*, vol. 153(2): pp. 226–239.

MacGregor, A. J., H. Snieder, A. S. Rigby, M. Koskenvuo, J. Kaprio, K. Aho, and A. J. Silman (2000): 'Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins'. *Arthritis and Rheumatism*, vol. 43(1): pp. 30–37.

Mahdi, Hiba, Benjamin A. Fisher, Henrik Källberg, Darren Plant, Vivianne Malmström, Johan Rönnelid, Peter Charles, Bo Ding, Lars Alfredsson, Leonid Padyukov, Deborah P. M. Symmons, Patrick J. Venables, Lars Klareskog, and Karin Lundberg (2009): 'Specific interaction between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis'. *Nature genetics*, vol. 41(12): pp. 1319–1324.

Mallat, Ariane, Fatima Teixeira-Clerc, Vanessa Deveaux, and Sophie Lotersztajn (2007): 'Cannabinoid receptors as new targets of antifibrosing strategies during chronic liver diseases'. *Expert opinion on therapeutic targets*, vol. 11(3): pp. 403–409.

Maradit-Kremers, Hilal, Cynthia S. Crowson, Paulo J. Nicola, Karla V. Ballman, Véronique L. Roger, Steve J. Jacobsen, and Sherine E. Gabriel (2005): 'Increased unrecognized coronary heart disease and sudden deaths in rheumatoid arthritis: a population-based cohort study'. *Arthritis and Rheumatism*, vol. 52(2): pp. 402–411.

Mariño, Guillermo, Mireia Niso-Santano, Eric H. Baehrecke, and Guido Kroemer (2014): 'Self-consumption: the interplay of autophagy and apoptosis'. *Nature reviews. Molecular cell biology*, vol. 15(2): pp. 81–94.

Massa, Federico, Giovanni Marsicano, Heike Hermann, Astrid Cannich, Krisztina Monory, Benjamin F. Cravatt, Gian-Luca Ferri, Andrei Sibaev, Martin Storr, and Beat Lutz (2004): 'The endogenous cannabinoid system protects against colonic inflammation'. *The Journal of clinical investigation*, vol. 113(8): pp. 1202–1209.

Matias, Isabel and Vincenzo Di Marzo (2007): 'Endocannabinoids and the control of energy balance'. *Trends in endocrinology and metabolism: TEM*, vol. 18(1): pp. 27–37.

Matsumoto, S., U. Müller-Ladner, R. E. Gay, K. Nishioka, and S. Gay (1996): 'Ultrastructural demonstration of apoptosis, Fas and Bcl-2 expression of rheumatoid synovial fibroblasts'. *The Journal of rheumatology*, vol. 23(8): pp. 1345–1352.

Mbvundula, Estery C., Rowena A. D. Bunning, and K. D. Rainsford (2006): 'Arthritis and cannabinoids: HU-210 and Win-55,212-2 prevent IL-1alpha-induced matrix degradation in bovine articular chondrocytes in-vitro'. *The Journal of pharmacy and pharmacology*, vol. 58(3): pp. 351–358.

McInnes, Iain B. and Georg Schett (2011): 'The pathogenesis of rheumatoid arthritis'. *The New England journal of medicine*, vol. 365(23): pp. 2205–2219.

Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, and D. R. Compton (1995): 'Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors'. *Biochemical pharmacology*, vol. 50(1): pp. 83–90.

Mechoulam, R. and Y. Gaoni (1965): 'A Total Synthesis of DL-Delta-1-Tetrahydrocannabinol, The Active Constituent of Hashish'. *Journal of the American Chemical Society*, vol. 87: pp. 3273–3275.

Mechoulam, R., A. Shani, H. Edery, and Y. Grunfeld (1970): 'Chemical basis of hashish activity'. *Science (New York, N.Y.)* Vol. 169(3945): pp. 611–612.

Meinecke, Ingmar, Antje Cinski, Anja Baier, Marvin A. Peters, Berno Dankbar, Aline Wille, Andreas Drynda, Heidi Mendoza, Renate E. Gay, Ronald T. Hay, Barbara Ink, Steffen Gay, and Thomas Pap (2007): 'Modification of nuclear PML protein by SUMO-1 regulates Fas-induced apoptosis in rheumatoid arthritis synovial fibroblasts'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104(12): pp. 5073–5078.

Mengshol, J. A., M. P. Vincenti, C. I. Coon, A. Barchowsky, and C. E. Brinckerhoff (2000): 'Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappa B - Differential regulation of collagenase 1 and collagenase 3'. *Arthritis and Rheumatism*, vol. 43(4): pp. 801–811.

Miossec, Pierre, Thomas Korn, and Vijay K. Kuchroo (2009): 'Interleukin-17 and type 17 helper T cells'. *The New England journal of medicine*, vol. 361(9): pp. 888–898.

Mitrirattanakul, Somsak, Navapoln Ramakul, Andre V. Guerrero, Yoshizo Matsuka, Takeshi Ono, Hirotate Iwase, Ken Mackie, Kym F. Faull, and Igor Spigelman (2006): 'Site-specific increases in peripheral cannabinoid receptors and their endogenous ligands in a model of neuropathic pain'. *Pain*, vol. 126(1-3): pp. 102–114.

Müller-Ladner, U., J. Kriegsmann, B. N. Franklin, S. Matsumoto, T. Geiler, R. E. Gay, and S. Gay (1996): 'Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice'. *The American journal of pathology*, vol. 149(5): pp. 1607–1615.

Munro, S., K. L. Thomas, and M. Abu-Shaar (1993): 'Molecular characterization of a peripheral receptor for cannabinoids'. *Nature*, vol. 365(6441): pp. 61–65.

Nishimoto, Norihiro, Tadamitsu Kishimoto, and Kazuyuki Yoshizaki (2000): 'Anti-interleukin 6 receptor antibody treatment in rheumatic disease'. *Annals of the Rheumatic Diseases*, vol. 59(Supplement 1): pp. i21–i27.

Nishimoto, Norihiro, Kazuyuki Yoshizaki, Nobuyuki Miyasaka, Kazuhiko Yamamoto, Shinichi Kawai, Tsutomu Takeuchi, Jun Hashimoto, Junichi Azuma, and Tadamitsu Kishimoto (2004): 'Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial'. *Arthritis and Rheumatism*, vol. 50(6): pp. 1761–1769.

Nissen, Steven E. et al. (2008): 'Effect of rimonabant on progression of atherosclerosis in patients with abdominal obesity and coronary artery disease: the STRADIVARIUS randomized controlled trial'. *JAMA*, vol. 299(13): pp. 1547–1560.

O'Dell, James R., Ted R. Mikuls, Thomas H. Taylor, Vandana Ahluwalia, Mary Brophy, Stuart R. Warren, Robert A. Lew, Amy C. Cannella, G. Kunkel, Ciaran S. Phibbs, Aslam H. Anis, Sarah Leatherman, and Edward Keystone (2013): 'Therapies for active rheumatoid arthritis after methotrexate failure'. *The New England journal of medicine*, vol. 369(4): pp. 307–318.

Ofek, Orr, Meliha Karsak, Nathalie Leclerc, Meirav Fogel, Baruch Frenkel, Karen Wright, Joseph Tam, Malka Attar-Namdar, Vardit Kram, Esther Shohami, Raphael Mechoulam, Andreas Zimmer, and Itai Bab (2006): 'Peripheral cannabinoid receptor, CB2, regulates bone mass'. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103(3): pp. 696–701.

Ohata, Junko, Nathan J. Zvaifler, Mitsufumi Nishio, David L. Boyle, Susan L. Kalled, Dennis A. Carson, and Thomas J. Kipps (2005): 'Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 174(2): pp. 864–870.

Oka, Saori, Junichi Wakui, Shinobu Ikeda, Shin Yanagimoto, Seishi Kishimoto, Maiko Gokoh, Miwako Nasui, and Takayuki Sugiura (2006): 'Involvement of the cannabinoid CB2 receptor and its endogenous ligand 2-arachidonoylglycerol in oxazolone-induced contact dermatitis in mice'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 177(12): pp. 8796–8805.

Pacher, Pál, Sándor Bátkai, and George Kunos (2006): 'The endocannabinoid system as an emerging target of pharmacotherapy'. *Pharmacological reviews*, vol. 58(3): pp. 389–462.

Pakozdi, Angela, Mohammad A. Amin, Christian S. Haas, Rita J. Martinez, G. Kenneth Haines, Lanie L. Santos, Eric F. Morand, John R. David, and Alisa E. Koch (2006): 'Macrophage migration inhibitory factor: a mediator of matrix metalloproteinase-2 production in rheumatoid arthritis'. *Arthritis research & therapy*, vol. 8(4): R132.

Patel, D. D., J. P. Zachariah, and L. P. Whichard (2001): 'CXCR3 and CCR5 ligands in rheumatoid arthritis synovium'. *Clinical immunology (Orlando, Fla.)* Vol. 98(1): pp. 39–45.

Pérez-García, Selene, Yasmina Juarranz, Mar Carrión, Irene Gutiérrez-Cañas, Andrew Margioris, José L. Pablos, Christos Tsatsanis, and Rosa P. Gomariz (2011): 'Mapping the CRF-urocortins system in human osteoarthritic and rheumatoid synovial fibroblasts: effect of vasoactive intestinal peptide'. *Journal of cellular physiology*, vol. 226(12): pp. 3261–3269.

Perlman, H., C. Georganas, L. J. Pagliari, A. E. Koch, K. Haines, and R. M. Pope (2000): 'Bcl-2 expression in synovial fibroblasts is essential for maintaining mitochondrial homeostasis and cell viability'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 164(10): pp. 5227–5235.

Pertwee, R. G., A. C. Howlett, M. E. Abood, S. P.H. Alexander, V. Di Marzo, Elphick, P. J. Greasley, H. S. Hansen, G. Kunos, K. Mackie, R. Mechoulam, and R. A. Ross (2010): 'International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid Receptors and Their Ligands: Beyond CB1 and CB2'. *Pharmacological reviews*, vol. 62(4): pp. 588–631.

Petrosino, Stefania, Enza Palazzo, Vito de Novellis, Tiziana Bisogno, Francesco Rossi, Sabatino Maione, and Vincenzo Di Marzo (2007): 'Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats'. *Neuropharmacology*, vol. 52(2): pp. 415–422.

Pongratz, Georg and Rainer H. Straub (2013): 'Role of peripheral nerve fibres in acute and chronic inflammation in arthritis'. *Nature reviews. Rheumatology*, vol. 9(2): pp. 117–126.

Rajesh, M., P. Mukhopadhyay, G. Haskó, J. W. Huffman, K. Mackie, and P. Pacher (2008): 'CB2 cannabinoid receptor agonists attenuate TNF-alpha-induced human vascular smooth muscle cell proliferation and migration'. *British journal of pharmacology*, vol. 153(2): pp. 347–357.

Remmers, Elaine F. et al. (2007): 'STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus'. *The New England journal of medicine*, vol. 357(10): pp. 977–986.

Richardson, Denise, Richard G. Pearson, Nisha Kurian, M. Liaque Latif, Michael J. Garle, David A. Barrett, David A. Kendall, Brigitte E. Scammell, Alison J. Reeve, and Victoria Chapman (2008): 'Characterisation of the cannabinoid receptor system in synovial tissue and fluid in patients with osteoarthritis and rheumatoid arthritis'. *Arthritis research & therapy*, vol. 10(2): R43.

Rosengren, Sanna, David L. Boyle, and G. S. Firestein (2007): 'Acquisition, culture, and phenotyping of synovial fibroblasts'. *Methods in molecular medicine*, vol. 135: pp. 365–375.

Ryberg, E., N. Larsson, S. Sjögren, S. Hjorth, N-O Hermansson, J. Leonova, T. Elebring, K. Nilsson, T. Drmota, and P. J. Greasley (2007): 'The orphan receptor GPR55 is a novel cannabinoid receptor'. *British journal of pharmacology*, vol. 152(7): pp. 1092–1101.

Sarker, Krishna Pada, Kamal Krishna Biswas, Munekazu Yamakuchi, Ki-Young Lee, Teruto Hahiguchi, Michael Kracht, Isao Kitajima, and Ikuro Maruyama (2003): 'ASK1-p38 MAPK/JNK signaling cascade mediates anandamide-induced PC12 cell death'. *Journal of neurochemistry*, vol. 85(1): pp. 50–61.

Sarnataro, Daniela, Simona Pisanti, Antonietta Santoro, Patrizia Gazzerro, Anna Maria Malfitano, Chiara Laezza, and Maurizio Bifulco (2006): 'The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits human breast cancer cell proliferation through a lipid raft-mediated mechanism'. *Molecular pharmacology*, vol. 70(4): pp. 1298–1306.

Schaible, Hans-Georg and Rainer H. Straub (2014): 'Function of the sympathetic supply in acute and chronic experimental joint inflammation'. *Autonomic neuroscience : basic & clinical*, vol. 182: pp. 55–64.

Schett, G. and G. S. Firestein (2010): 'Mr Outside and Mr Inside: classic and alternative views on the pathogenesis of rheumatoid arthritis'. *Annals of the rheumatic diseases*, vol. 69(5): pp. 787–789.

Schett, G., M. Tohidast-Akrad, J. S. Smolen, B. J. Schmid, C. W. Steiner, P. Bitzan, P. Zenz, K. Redlich, Q. Xu, and G. Steiner (2000): 'Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 43(11): pp. 2501–2512.

Scott, David L., Frederick Wolfe, and Tom W. J. Huizinga (2010): 'Rheumatoid arthritis'. *Lancet*, vol. 376(9746): pp. 1094–1108.

Sharir, Haleli and Mary E. Abood (2010): 'Pharmacological characterization of GPR55, a putative cannabinoid receptor'. *Pharmacology & therapeutics*, vol. 126(3): pp. 301–313.

Silvestri, Cristoforo and Vincenzo Di Marzo (2013): 'The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders'. *Cell metabolism*, vol. 17(4): pp. 475–490.

Sokka, Tuulikki, Glenn Haugeberg, Juha Asikainen, Inger Johanne Widding Hansen, Arto Kokko, Tuomas Rannio, Dag Magnar Soldal, and Pekka Hannonen (2013): 'Similar clinical outcomes in rheumatoid arthritis with more versus less expensive treatment strategies. Observational data from two rheumatology clinics'. *Clinical and experimental rheumatology*, vol. 31(3): pp. 409–414.

Starowicz, Katarzyna, Santosh Nigam, and Vincenzo Di Marzo (2007): 'Biochemistry and pharmacology of endovanilloids'. *Pharmacology & therapeutics*, vol. 114(1): pp. 13–33.

Stefanski, Volker, Susanne K. Hemschemeier, Kerstin Schunke, Anja Hahnel, Christine Wolff, and Rainer H. Straub (2013): 'Differential effect of severe and moderate social stress on blood immune and endocrine measures and susceptibility to collagen type II arthritis in male rats'. *Brain, behaviour and immunity*, vol. 29: pp. 156–165.

Stolt, Patrik, Abqariyah Yahya, Camilla Bengtsson, Henrik Kallberg, Johan Ronnelid, Ingvar Lundberg, Lars Klareskog, and Lars Alfredsson (2010): 'Silica exposure

among male current smokers is associated with a high risk of developing ACPA-positive rheumatoid arthritis'. *Annals of the rheumatic diseases*, vol. 69(6): pp. 1072–1076.

Storr, Martin A. and Keith A. Sharkey (2007): 'The endocannabinoid system and gut-brain signalling'. *Current opinion in pharmacology*, vol. 7(6): pp. 575–582.

Straub, R. H., M. Cutolo, F. Buttgereit, and G. Pongratz (2010): 'Energy regulation and neuroendocrine-immune control in chronic inflammatory diseases'. *Journal of internal medicine*, vol. 267(6): pp. 543–560.

Straub, Rainer H. (2007): *Lehrbuch der klinischen Pathophysiologie komplexer chronischer Krankheiten*. Psycho-neuro-endokrino-Immunologie. Göttingen: Vandenhoeck & Ruprecht.

– (2014): 'Interaction of the endocrine system with inflammation: a function of energy and volume regulation'. *Arthritis research & therapy*, vol. 16(1): p. 203.

Takemura, S., A. Braun, C. Crowson, P. J. Kurtin, R. H. Cofield, W. M. O'Fallon, J. J. Goronzy, and C. M. Weyand (2001): 'Lymphoid neogenesis in rheumatoid synovitis'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 167(2): pp. 1072–1080.

Tan, Y., J. Rouse, A. Zhang, S. Cariati, P. Cohen, and M. J. Comb (1996): 'FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2'. *The EMBO journal*, vol. 15(17): pp. 4629–4642.

Taylor, Peter C. and Bran Sivakumar (2005): 'Hypoxia and angiogenesis in rheumatoid arthritis'. *Current opinion in rheumatology*, vol. 17(3): pp. 293–298.

ter Wee, M. M., W. F. Lems, H. Usan, A. Gulpen, and A. Boonen (2012): 'The effect of biological agents on work participation in rheumatoid arthritis patients: a systematic review'. *Annals of the rheumatic diseases*, vol. 71(2): pp. 161–171.

Tolboom, Tanja C.A., Annette H.M. van der Helm-Van Mil, Rob G.H.H. Nelissen, Ferdinand C. Breedveld, Rene E.M. Toes, and Tom W.J. Huizinga (2005): 'Invasiveness of fibroblast-like synoviocytes is an individual patient characteristic associated with the rate of joint destruction in patients with rheumatoid arthritis'. *Arthritis and rheumatism*, vol. 52(7): pp. 1999–2002.

van der Helm-van Mil, Annette H. M. and Tom W. J. Huizinga (2008): 'Advances in the genetics of rheumatoid arthritis point to subclassification into distinct disease subsets'. *Arthritis research & therapy*, vol. 10(2).

van der Linden, Michael P. M., Diane van der Woude, Andreea Ioan-Facsinay, Levarht, E W Nivine, Gerrie Stoeken-Rijsbergen, Huizinga, Tom W J, Toes, René E M, and van der Helm-van Mil, Annette H M (2009): 'Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 60(8): pp. 2232–2241.

Vanden Berghe, Tom, Andreas Linkermann, Sandrine Jouan-Lanhouet, Henning Walczak, and Peter Vandenabeele (2014): 'Regulated necrosis: the expanding network of non-apoptotic cell death pathways'. *Nature reviews. Molecular cell biology*, vol. 15(2): pp. 135–147.

Villiger, P. M., R. Terkeltaub, and M. Lotz (1992): 'Production of monocyte chemoattractant protein-1 by inflamed synovial tissue and cultured synoviocytes'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 149(2): pp. 722–727.

Watanabe, Nobuyuki, Kiichiro Ando, Shinichi Yoshida, Sawako Inuzuka, Masaaki Kobayashi, Nobuo Matsui, and Takashi Okamoto (2002): 'Gene expression profile analysis of rheumatoid synovial fibroblast cultures revealing the overexpression of genes responsible for tumor-like growth of rheumatoid synovium'. *Biochemical and biophysical research communications*, vol. 294(5): pp. 1121–1129.

Wegner, Natalia, Robin Wait, Aneta Sroka, Sigrun Eick, Ky-Anh Nguyen, Karin Lundberg, Andrew Kinloch, Shauna Culshaw, Jan Potempa, and Patrick J. Venables (2010): 'Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen

and a-enolase: implications for autoimmunity in rheumatoid arthritis'. *Arthritis and Rheumatism*, vol. 62(9): pp. 2662–2672.

Westra, J., P. C. Limburg, P. de Boer, and M. H. van Rijswijk (2004): 'Effects of RWJ 67657, a p38 mitogen activated protein kinase (MAPK) inhibitor, on the production of inflammatory mediators by rheumatoid synovial fibroblasts'. *Annals of the rheumatic diseases*, vol. 63(11): pp. 1453–1459.

Widmann, C., S. Gibson, M. B. Jarpe, and G. L. Johnson (1999): 'Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human'. *Physiological reviews*, vol. 79(1): pp. 143–180.

Xing, J., D. D. Ginty, and M. E. Greenberg (1996): 'Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase'. *Science (New York, N.Y.)* Vol. 273(5277): pp. 959–963.

Yamanishi, Yuji, David L. Boyle, Melody Clark, Rich A. Maki, Micky D. Tortorella, Elizabeth C. Arner, and G. S. Firestein (2002): 'Expression and regulation of aggrecanase in arthritis: the role of TGF-beta'. *Journal of immunology (Baltimore, Md. : 1950)*, vol. 168(3): pp. 1405–1412.

Yeoh, Nigel, Jeremy P. Burton, Praema Suppiah, Gregor Reid, and Simon Stebbings (2013): 'The role of the microbiome in rheumatic diseases'. *Current rheumatology reports*, vol. 15(3): p. 314.

Ziegler, Stefanie, Dörte Huscher, Kirsten Karberg, Andreas Krause, Siegfried Wassenberg, and Angela Zink (2010): 'Trends in treatment and outcomes of rheumatoid arthritis in Germany 1997-2007: results from the National Database of the German Collaborative Arthritis Centres'. *Annals of the rheumatic diseases*, vol. 69(10): pp. 1803–1808.

Curriculum Vitae

Persönlich

Name	Martin Apitz geboren am 09.09.1987 in Schkeuditz Bergstraße 69, 69121 Heidelberg martin.apitz@med.uni-heidelberg.de

Schulische Ausbildung

1999-2007	Abitur, Stadtgartengymnasium Saarlouis

Akademische Ausbildung

2007-2013	Universität Regensburg
2009	1. Abschnitt der ärztlichen Prüfung
2013	2. Abschnitt der ärztlichen Prüfung

Berufsstelle

05/2014 - heute	Assistenzarzt an der Chirurgischen Klinik, Universitätsklinikum Heidelberg

Stipendien

2009-2013	Stipendiat der Studienstiftung des deutschen Volkes

Eidesstattliche Erklärung

Die Dissertation wurde von Herrn Prof. Dr. med. Rainer Straub und Herrn Dr. med. Torsten Lowin angeregt und überwacht.
Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt

Heidelberg Martin Apitz

Danksagung

Zuerst geht mein Dank an Prof. Dr. Rainer Straub für die Überlassung des Themas. Ich bedanke mich bei Dr. Torsten Lowin für den steten Ratschlag bei Problemen, für die Anregungen für weitere Experimente sowie die kritische Überprüfung der Ergebnisse. Zuletzt danke ich dem ganzen Team der DFG Research Unit FOR696, besonders bei Tanja Späth, Olga Wiesner, Dagmar Halbritter, Madlen Melzer und Luise Rauch für die methodische Einarbeitung und stete Geduld.
Mein größter Dank gilt meinen Eltern. Ich bin stolz auf euch.