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„The Transcription Factor FoxO3a differentially regulates the expression of proinflammatory genes in rheumatoid fibroblast-like synoviocytes“

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1 Abstract

English:

The transcription factor (TF) FoxO3a is known to integrate information from multiple upstream signals (e.g., cytokines, oxidative stress, growth factors) in order to maintain tissue homeostasis during stress. Recently, an association between a single-nucleotide polymorphism (SNP) in FO3a and the severity of rheumatoid arthritis (RA) was reported. The role of FoxO3a in fibroblast-like synoviocytes (FLS), which are known to be key effector cells in RA, has, however, not yet been investigated. We here show that the pro-inflammatory cytokine TNFα promotes phosphorylation and cytoplasmic shuttling of FoxO3a in RA-FLS. RNA-sequencing of RA-FLS that were transfected with FoxO3a targeting siRNA revealed that FoxO3a controls a number of pro-inflammatory genes such as CXCL9, CXCL10, CXCL11, and MMP3. Thus, our studies demonstrate that FoxO3a plays a crucial role for the TNFα-driven inflammatory response in FLS.

Deutsch:

Der Transkriptionsfaktor (TF) FoxO3a dient als Transmitter für unterschiedliche Signale (Zytokine, oxidativer Stress, Wachstumsfaktoren), um unter Belastungsreaktionen die Gewebshomöostase aufrecht zu erhalten. Vor kurzem wurde über eine Verbindung zwischen einem Einzelnukleotid-Polymorphismus (SNP) in FoxO3a und der Schwere der Erkrankung bei rheumatoider Arthrithis berichtet. Die Rolle von FoxO3a in fibroblastenartige Synoviozyten (FLS), welche als Schlüssel Faktoren in der RA bekannt sind, wurden bisher allerdings noch nicht untersucht. Wir zeigen, dass das pro-inflammatorische Zytokin TNFα die Phosphorylierung und den Zytoplasmatischen Transfer von FoxO3a begünstigt. RNA-Sequenzierung von RA-FLS, welche mit si-RNA, die gegen FoxO3a gerichtet war, transfiziert wurden, offenbarte eine Reihe von FoxO3a regulierten pro-inflammatorischen Genen, wie CXCL9, CXCL10, CXCL11 und MMP3 . Unsere Untersuchungen zeigen, dass FoxO3a für die TNFα induzierte Entzündungsreaktion von FLS von entscheidender Bedeutung ist; es erlaubt den FLS das entzündliche Geschehen zu verstärken und den entzündlich destruierenden Prozess voranzutreiben.

Introduction

1.1 Organization and function of the synovial membrane

The synovium (also known as synovial membrane) is a highly specialized tissue, which is localized at the inner side of the joint-capsule. The synovium is built up by two layers, an intima (lining) layer and a subintima (sublining) layer. The sublining layer consists of scattered blood vessels, fat cells and fibroblast-like synoviocytes (FLS) and is characterized by a very low density of cells. Two types of synoviocytes can be found in the intimate lining layer, namely FLS and macrophage-like synovial cells (MLS).
MLS show a similar phenotype to other tissue resident macrophage populations, including the expression CD11b, CD14, CD68, CD163, MHC class II and FcR_{γ}. They express markers of hematopoietic origin and show phagocytic activity. FLS are cells of mesenchymal origin and express type IV and V collagens, vimentin, and CD90. In contrast to other fibroblast lineages, FLS display specific features such as the expression of the adhesion molecule cadherin-11, which plays a key role in homotypic aggregation of FLS in vitro and in vivo [2, 3]. The expression of uridine diphosphoglucose dehydrogenase (UDPGD) by the intimal lining reflects the ability to synthesize hyaluronic acid, a major component of the synovial fluid and extracellular matrix [2]. Equal in their distribution, FLS and MLS form a compact layer, two to three cells deep, that provides nutrients to support the avascular articular cartilage and provides structural stability through the formation of extracellular matrix. The synovial lining lacks epithelial cells, an organized basement membrane, tight junctions, and desmosomes, rather the lining cells are embedded in the specialized extracellular matrix which provides a moderate level of structural stability [1]. Together, the synovium supports the articular cartilage by providing nutrients as well as producing lubricating synovial fluid [2].

1.2 The synovial membrane in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease which causes progressive joint destruction and functional disability. RA is associated with an increased prevalence of several comorbidities, such as cancer and cardiovascular diseases [1]. The initiative cause of RA remains elusive. Environmental cues, such as smoking, and genetic or epigenetic variations have been implicated as important risk factors for RA development and severity of the disease [32]. Thus, genetic and environmental factors contribute to perturbations in the immune system, which result in persistent inflammation of diarthrodial joints. In RA, the synovium transforms into a hyperplastic, invasive tissue. The lining layer expands from a 1-2 up to a 10-20 cells thick layer consisting of both, MLS and FLS [3]. In RA, FLS and MLS form the so called “pannus” that invades and destroys the articular cartilage and the subchondral bone. FLS and MLS also produce vast amounts of cytokines and chemokines that activate T-cells, B-cells and other immune cells. Moreover, FLS and MLS produce matrix metallo-proteinases (MMPs) that promote tissue destruction [4]. During the course of RA the inflamed sublining layer becomes infiltrated by leucocytes, such as T-cells and B-cells [2]. Also, due to the hypoxic inflammatory microenvironment, neo-angiogenesis occurs [3].

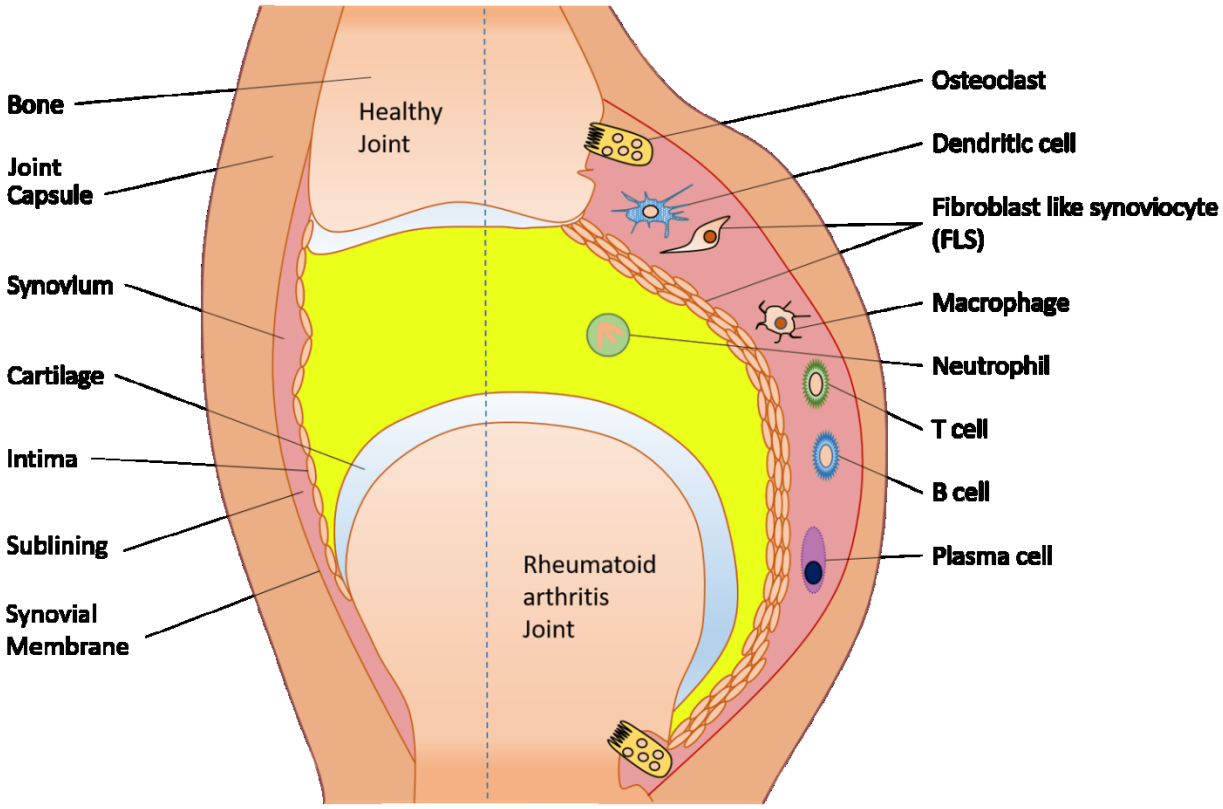

Figure 1: Schematic overview of a joint under healthy and RA conditions

The synovial environment in RA is highly genotoxic due to the abundant reactive nitrogen and oxygen species which ultimately favour apoptotic cellular stress levels. Interestingly, RA-FLS seem to be protected from apoptosis in vivo. The Bcl-2 family proteins, responsible for the intrinsic apoptosis pathway, are differentially expressed in the RA synovium compared to OA [3]. The increased expression of the anti-apoptotic Bcl-2 and Mcl-1 in the lymphoid aggregates suggests protection of synovial B- and T-cells [5, 9]. Also, the extrinsic pathway of apoptosis, including the ligand-bound receptors TNF-R1, Fas/CD95 and TRAIL-R1 and –R2 seem to be counteracted via several influences. Han et al. for instance demonstrated that NFκB, which is highly activated in lining cells in RA, provides a strong anti-apoptotic signal and links inflammation to decreased apoptosis [6]. Further, post-translational modification plays a crucial role in the avoidance of apoptosis. Phosphatase and tensin homolog (PTEN), which is known to dephosphorylate phosphoinositide 3-kinase (PI3K) substrates, for example is very low in the synovial intima, consequence to NFκB activation which ultimately favours cell survival [7]. Along with their resistance to apoptosis, FLS also have been reported to actively proliferate in this cytotoxic environment [1]. Therefore, FLS can be described to counteract the cytotoxic environment in a diametric way.

1.3 FLS are Key effector cells in RA

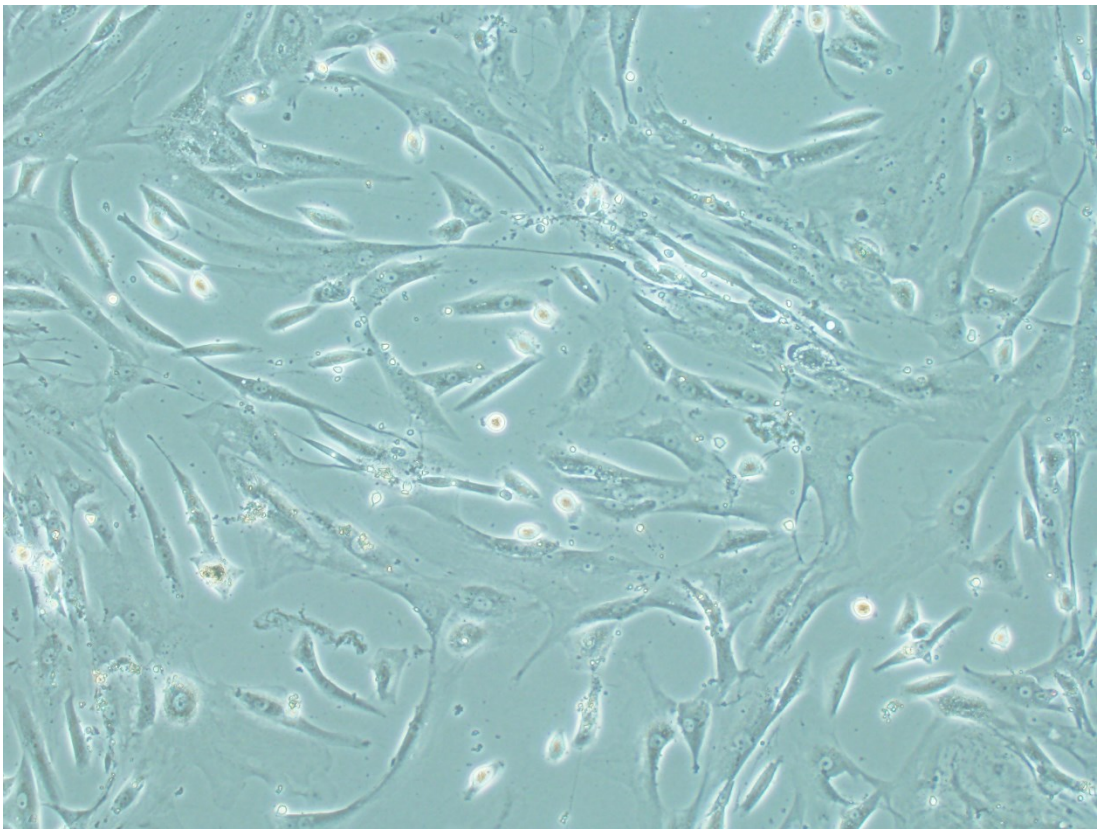

Figure 2: RA-fibroblast like synoviocytes (RA-FLS) in culture

FLS are spindle shaped cells of mesenchymal origin. Together with MLS, FLS form the synovial lining layer. FLS are important for the maintenance of synovial lining flexibility and integrity as well as for the synthesis of numerous lubricating substances. FLS are also crucial for cartilage nutrition. In RA, FLS display an aggressive, invasive phenotype that contributes to immune cell recruitment and actively participate in joint destruction [4, 40]. The polyarticular affection, which is typical for RA, seems to be a result of the migration of invasive FLS through the bloodstream, which has been shown in SCID mice by Lefêvre et al [53]. Especially, pro-inflammatory cytokines, such as TNFα or IL-1, have been shown to play a crucial role in the activation of RA-FLS [8, 10, 19]. In addition, intrinsic events, such as epigenetic modifications in the genome or single nucleotide modifications may play an important role in FLS based on the persistence of inflammation [10]. For example, histone-deacetlyases (HDACs) levels have been reported to be reduced in RA patients, which suggest an increase in histone modifications [10]. CXCL12, a chemokine responsible for the development of pseudo-emperipolesis, is reported to be hypo-methylated and, therefore, highly expressed by RA-FLS [9, 10]. However, the exact role of epigenetic modifications in RA inflammation has not been fully unveiled yet.

FLS do not just act as passive responders to the immunoregulatory signal cascades in the joint; they also actively participate in the regulation of chronic inflammation. Cell-to-cell contact, antigen presentation, in addition to their very broad array of secreted mediators and stimulation of Toll-like receptors (TLRs 1 through 6) make them remarkably competent activators and regulators of the innate as well as the adaptive immune system [11]. For instance, activated FLS secrete SDF-1, which inhibits T-cell apoptosis, and thereby promotes the local T-cell response. Other FLS derived chemokines, such as the CXCR3-binding chemokines CXCL9, CXCL10, CXCL11 sustain inflammatory cell recruitment in rheumatoidsynovitis [60]. In addition to the recruiting and stimulatory features of FLS, RA-FLS also provide distinct microdomains for T-cells within the inflamed synovial tissue, by CXCL12 driven pseudo-emperipolesis [9]. FLS also produce the B-cell survival factor (BAFF/ TNFSF13B), which supports survival of B-cells and autoantibody production [4]. Increased expression of IL6 and granulocyte macrophage colony-stimulating factor (MG-CSF) by RA-FLS, further promotes the local expansion and activation of macrophages [4]. RA-FLS have also been reported to recruit monocytes and macrophages via the secretion of RANTES, IP-10, ENA-78, MCP-1, MIP-1α and MIP-1β in response to IL-1 or TNFα stimulation [4].

Despite their TNFα induced activation, FLS can also be stimulated by TLR-agonists. Especially TLR2, TLR3 and TLR4 are highly expressed in RA-FLS. The TLR-agonists, hyaluronan, fibrinogen in addition to exogenous bacterial peptidoglycan are commonly found in the RA synovial fluid and can potentially activate TLR2 and TLR4 [11, 41].TLR2, has been reported to induce the expression of various CXC-motif genes in RA-FLS when stimulated with peptidoglycan [17]. TLR3 in contrast, known as a double stranded RNA specific receptor, can also be activated by necrotic synovial fluid cells [10, 12]. Lipopolysaccharides, peptidoglycan and double stranded RNA (poly I:C) are the three key TLR ligands in FLS and induce production of pro-inflammatory cytokines. Interestingly, the interferon response linked to TLR3 signalling, is a potent stimulator of IL-6, MMP1 and MMP3 in RA-FLS [12]. TLR3 signalling in FLS involves the activation of IKKε and TBK1 which will then phosphorylate and activate the interferon regulatory factors (IRFs), especially IRF3 [12, 13]. Noteworthy, TNFα promotes IRF3 phosphorylation and therefore IRF3-mediated gene expression in FLS, a unique function in synoviocytes [4]. TLRs are generally known to sense for pathogen-associated molecule patterns (PAMPs), but it has also been reported that the TLR system in RA is also able to sense the damage-associated molecular patterns (DAMPs). For example, TLR3 accepts sensing the dsRNA from necrotic cells [10]. Moreover, it has been shown that peptidoglycans and bacterial DNA are retained in the RA synovia, resulting in a constant activation of TLR pathways [52]. These features underline the impact of TLR signalling in the progression of RA.

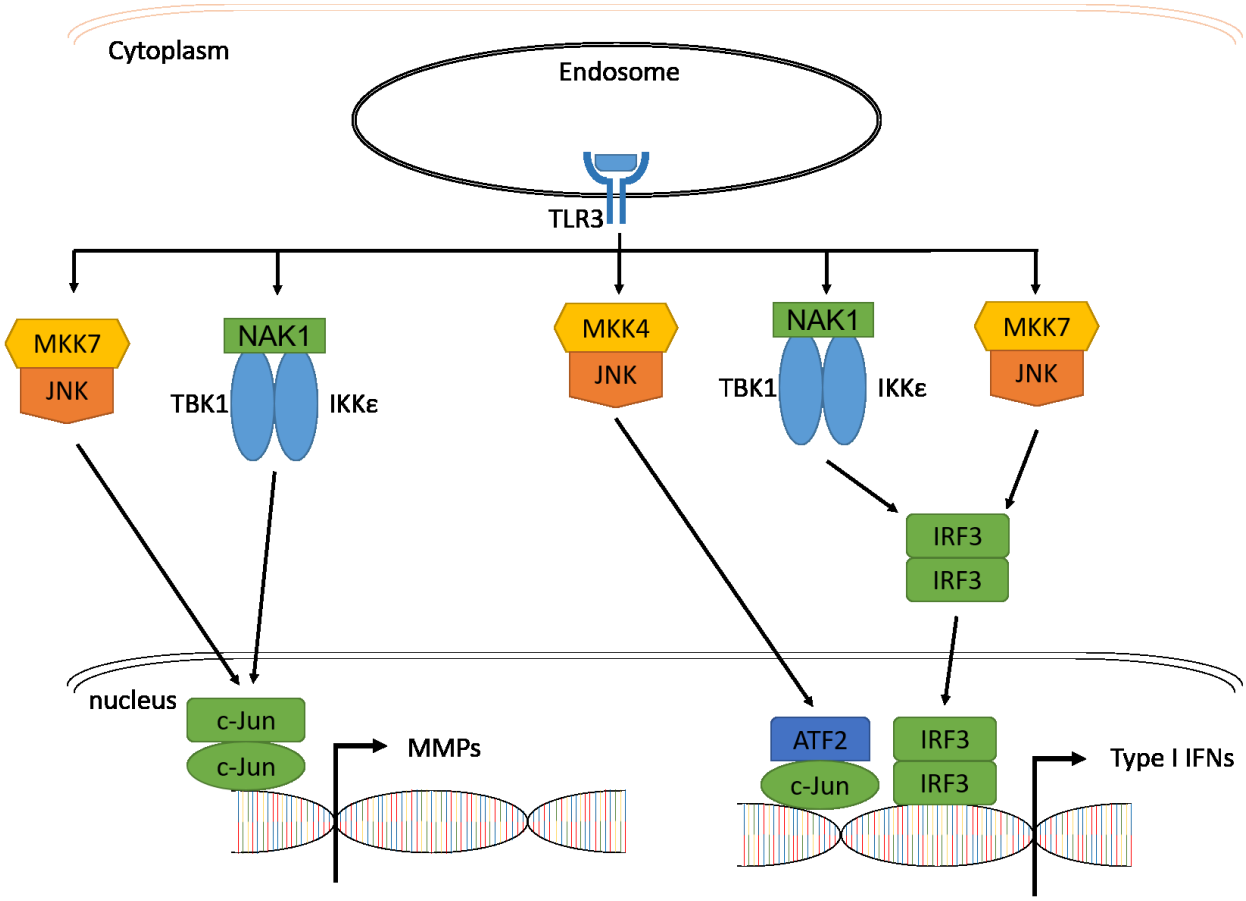

Figure 3: Simplified TLR3 Signalling in RA-FLS

Additional to their immunoregulatory features in RA, activated FLS also produce MMPs. Collagenases (MMP-1, MMP-13) and stromelysins (MMP-3) are abundantly expressed in the inflamed rheumatoid synovium. Increased expression of these tissue degrading enzymes contributes to connective tissue and cartilage destruction in RA [10, 39]. MMP expression is mainly regulated on the transcriptional level and induced by several factors like pro-inflammatory cytokines (e.g. TNFα), growth factors and reactive oxygen species [14]. Activator protein-1 (AP-1) is a family of transcription factors that has been reported to be the key promoter for MMP gene expression [5].RA-FLS also abundantly express numerous adhesion molecules, like the integrin α5/β1. These adhesion molecules are essential for anchoring RA-FLS in the extracellular matrix (ECM), e.g. the cartilage. Integrins interact with collagen, fibronectin and oligomeric matrix proteins. Ligation of these receptors activates multiple intracellular signalling pathways, like the MAPK-pathway, which results in increased expression of MMPs, suggesting that the ECM modulates its own destruction in RA [4, 10]. Besides, FLS are also the primary source of IL-6 and the granulocyte macrophage colony-stimulating factor (GM-CSF), which are highly abundant in RA synovitis and may contribute to the local expansion and activation of macrophages [4].

The chemokine system in RA has a complex composition, consisting of various chemoattractants for innate as well as adaptive immune cells; it shapes and regulates the cellular composition of the RA synovium. For instance, CCR5 and CXCR3 expression on T cells as well as MIP-1α and RANTES expression by FLS cause a predominant CD4+ Th1 T-cell phenotype. Furthermore the SDF-1 and CXCL16 expression of FLS supports the accumulation of CD4+ memory T-cells [1]. RA-FLS have also been reported to recruit monocytes and macrophages via the secretion of RANTES, IP-10, ENA-78, MCP-1, MIP-1α and MIP-1β in response to IL-1 or TNFα stimulation [4, 16]. Besides to this cytokine-driven chemokine production, FLS are also expressing chemokines in response to TLR stimulation. Especially the activation of the MyD88 pathway, resulting from TLR2 and TLR4 signalling stimulates FLS to produce chemokines [17]. Also the mentioned IRF3 pathway seems to play an important role in the chemokine production [12, 13]. Overall these data highlight the importance of FLS for the persistence of synovial inflammation and tissue destruction in RA.

1.4 Pathways that are activated in RA

Several signalling pathways are known to contribute to the aggressive behaviour of RA-FLS. Especially the MAPK-, the NFκB- and the JAK-STAT pathways have emerged as major FLS-activating signalling circuits. The mitogen-activated protein kinases (MAPK; c-Jun N-terminal kinase (JNK), p38 and the extra-cellular signal-regulated kinase (ERK) are evolutionary conserved and control a variety of cellular functions, such as metabolism, proliferation, apoptosis and cell differentiation. The activation of MAPKs is conserved and involves the several steps, including the cascaded activation of the MKKs (MAPK kinase kinases). The functions of each MAPK contain both, partially overlapping features but also unique ones. ERK for example, has a predominant role in regulating mitotic responses, cell differentiation and cell growth while p38 and JNK are the main responders to stress signals [15].In response to pro-inflammatory cues MAPKs-pathway control the expression of pro-inflammatory cytokines, chemokines and tissue-degrading enzymes (MMPs) in RA-FLS [43]. In response, principal components of the MAPK-pathways are activated in RA-FLS [4].

The NFκB family consists of five members: RelA (p65), RelB, c-REl, p50/p105 and p52/p100 which form homo/heterodimers. Under resting conditions they residue as an inactive complex with bound IκB in the cytoplasm. IKKα and IKKβ are two kinases that phosphorylate IκB and ultimately activate the Transcription factor NFκB.

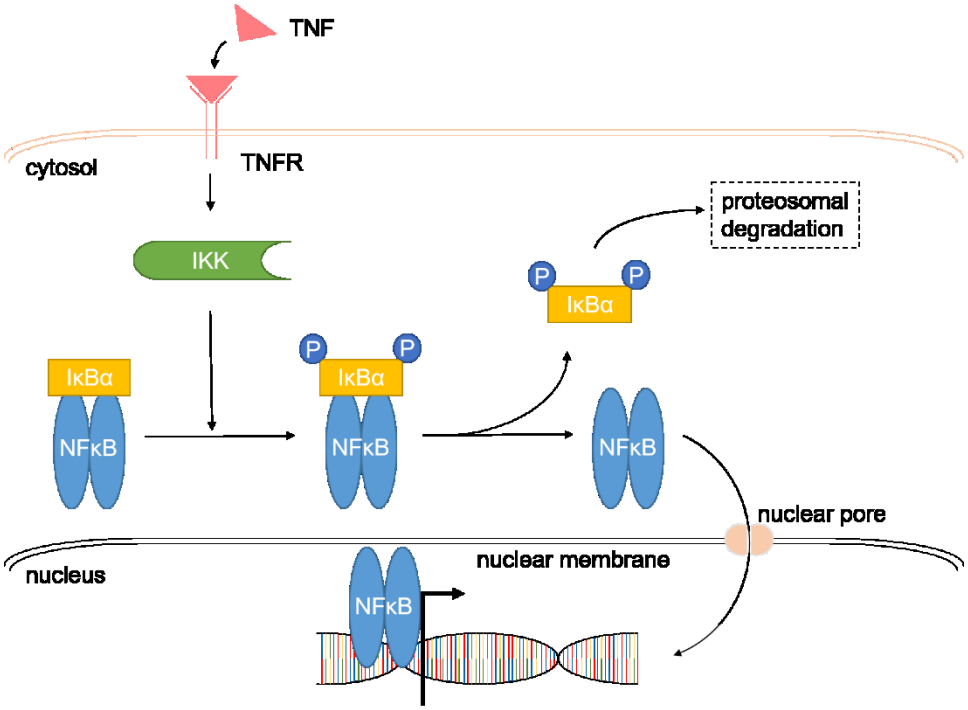

Figure 4: Simplified NFκB activation by IKK degradation

The phosphorylation of IκB will mark it for ubiquitination. Releasing of the previously IκB masked nuclear localization sequence on NFκB, causing the nuclear migration of the dimer. NFκB activation in RA has been shown by Aupperle et al, based on the expression of p50/p65 in the intimal lining cells under TNFα stimuli. IKKα and IKKβ are constitutively expressed in FLS and their kinase activity is increased within minutes of cytokine exposure [18]. In response to the classical cytokines in RA, RA-FLS are the primary source of IL-6 in the inflamed synovial tissue, which has been shown in an in-situ hybridization experiment by Brennan et al.[42]. Also cultured FLS spontaneously produce IL-6, which could be remarkably increased via IL-1 or TNFα stimulation due to an NFκB dependent pathway [18].

Several pro-inflammatory cytokines that are known to contribute to RA pathogenesis (e.g. IL6 or IFNγ) activate the JAK-STAT signalling pathway. The JAK-STAT signalling pathway is involved in various cellular processes such as cell death, cell division or antiviral response. The JAK-STAT signalling pathway consists of three key parts: the receptor, Janus kinases (JAKs) and signal transducer and activator of transcription proteins (STATs). There are four JAKs (JAK1, JAK2, JAK3 and TYK2) and six STATs (STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6). Binding of a ligand to its receptor causes phosphorylation of the receptor-bound JAKs. Activated JAKs then induce phosphorylation and activation of STATs. Activated STATs then form hetero- or homodimers and translocate into nucleus to initiate the transcription. Several JAKs (JAK1, JAK3) and STATs (STAT1, STAT3) are abundantly expressed in the inflamed, rheumatoid synovial tissues [57, 58]. Therefore it is not surprising, that inhibitors of JAKs, such as baricitinib or tofacitinib, are very effective drugs to treat RA [58, 60]. Although, JAK-inhibitors were developed to target immune cell activation, it was recently shown that these compounds also suppress RA-FLS activation (chemokine expression, FLS migration and invasion) [59].

1.5 The FoxO family

The Forkhead box O (FoxO) family consists of four members, namely FoxO1, FoxO3a, FoxO4 and FoxO6. FoxOs are conserved transcription factors that share the same consensus sequence (5'-TTGTTTAC-3') [20].

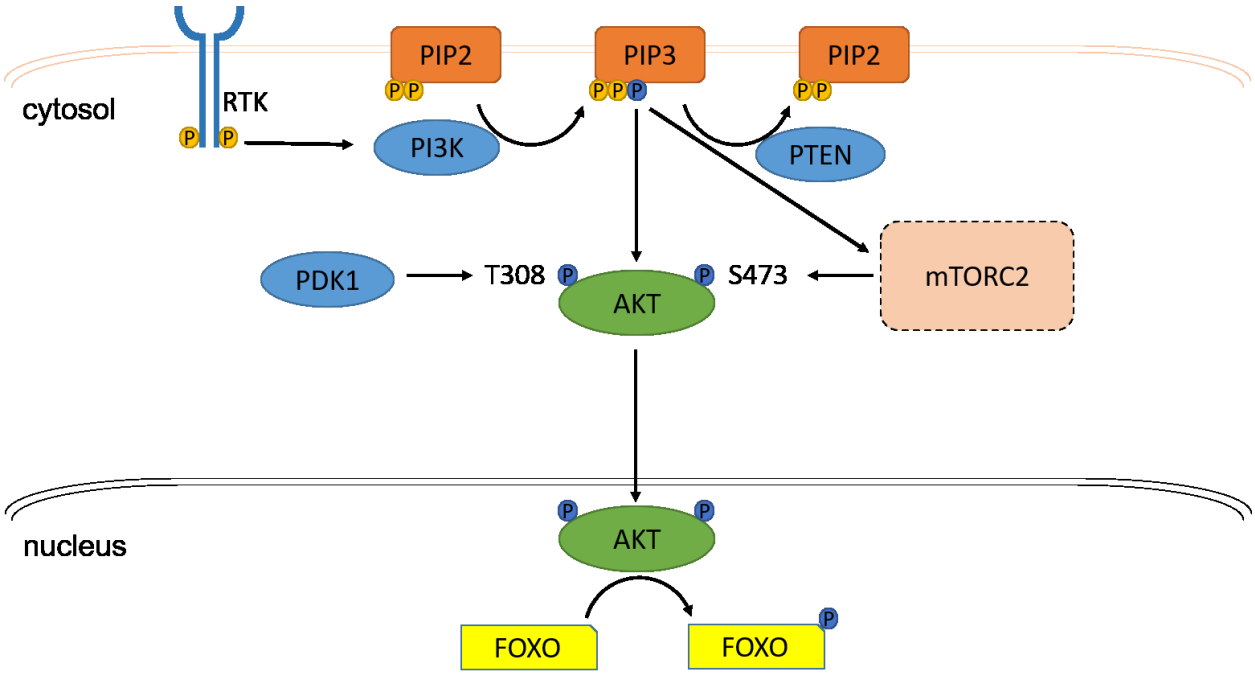

Figure 5: Simplified overview of PI3K – AKT driven FoxO phosphorylation

Members of the Forkhead box Family O are broadly expressed in mammalians, but the expression of the isoforms seems to be in tissue-specific pattern. FoxO1 is mainly found in adipose tissue, uterus and ovaries [44]. FoxO3a is expressed in most tissues but particularly high in brain, spleen, heart and ovaries [21]. FoxO4 is highly expressed in skeletal muscle, cardiac muscle and adipose tissue. FoxO6 is interestingly only expressed in the adult brain [21]. FoxOs are known to regulate numerous important cellular functions, such as cell cycle arrest, T-Cell mediated tolerance, glucose metabolism and longevity [19, 20].

1.6 Pathways that regulate FoxO activity

According to our current understandings, FoxOs are important signalling integrators for the maintenance of homeostasis under various stress conditions [19,20,21,23]. FoxOs are generally kept in an inactive state under physiological conditions. Growth factors, like EGF or insulin are the best known negative regulators of FoxO3a activity. This requires the activation of the PI3K-AKT signalling pathway [54]. Upon PI3K activation, active (phosphorylated) AKT translocate to the nucleus and phosphorylates FoxO at different residues, depending on the isoform. In the case of FoxO3a, the phosphorylation sites are located at T32, S253 and S315. This phosphorylation results in the binding of FoxO to 14-3-3, which promotes nuclear exclusion of the FoxO – 14-3-3 complex [22]. Under conditions of stress, the concentration of nuclear FoxOs recovers partially by re-localization and partially by de novo synthesis [36]. The nuclear re-localization is mediated via the MAPK JNK, which has been shown initially in drosophila melanogaster, where JNK inhibits insulin signalling and induces the FoxO-14-3-3 release [23]. Nuclear translocation of FoxO results in increased transcription activity of FoxO and leads to increased expression of FoxO targeted genes. FoxO activity is primary determined by the protein levels of FoxOs, which are reported to be regulated by ubiquitin mediated degradation followed to the cytoplasmic shuttling [24]. Known E3 ligases for FoxO proteins include SKP2 which binds to AKT-phosphorylated FoxOs and MDM2, which binds ERK-phosphorylated FoxOs and can induce both, mono-ubiquitination as well as poly-ubiquitination [25,45]. Poly-ubiquitination targets FoxO for degradation whereas mono-ubiquitination of FoxO3a, for example, results in an increased nuclear abundance of FoxO3a and therefore in an increased transcriptional activity [26].

1.7 Posttranslational modification – The FoxO code

As mentioned previously FoxOs localization and activity are also regulated by their post translational modifications. The large variety of possible posttranslational modifications (PTM) of transcription factors led to the suggestion that codes of various PTMs exist to regulate the activity in a more detailed way[21]. Wand et al. suggests that this code might determine the binding of co-factors to assure an appropriate transcriptional regulation [27]. They further noted that the C-terminal transactivation domain is intrinsic disordered supporting the idea of a FoxO-code induced-fit model [27].

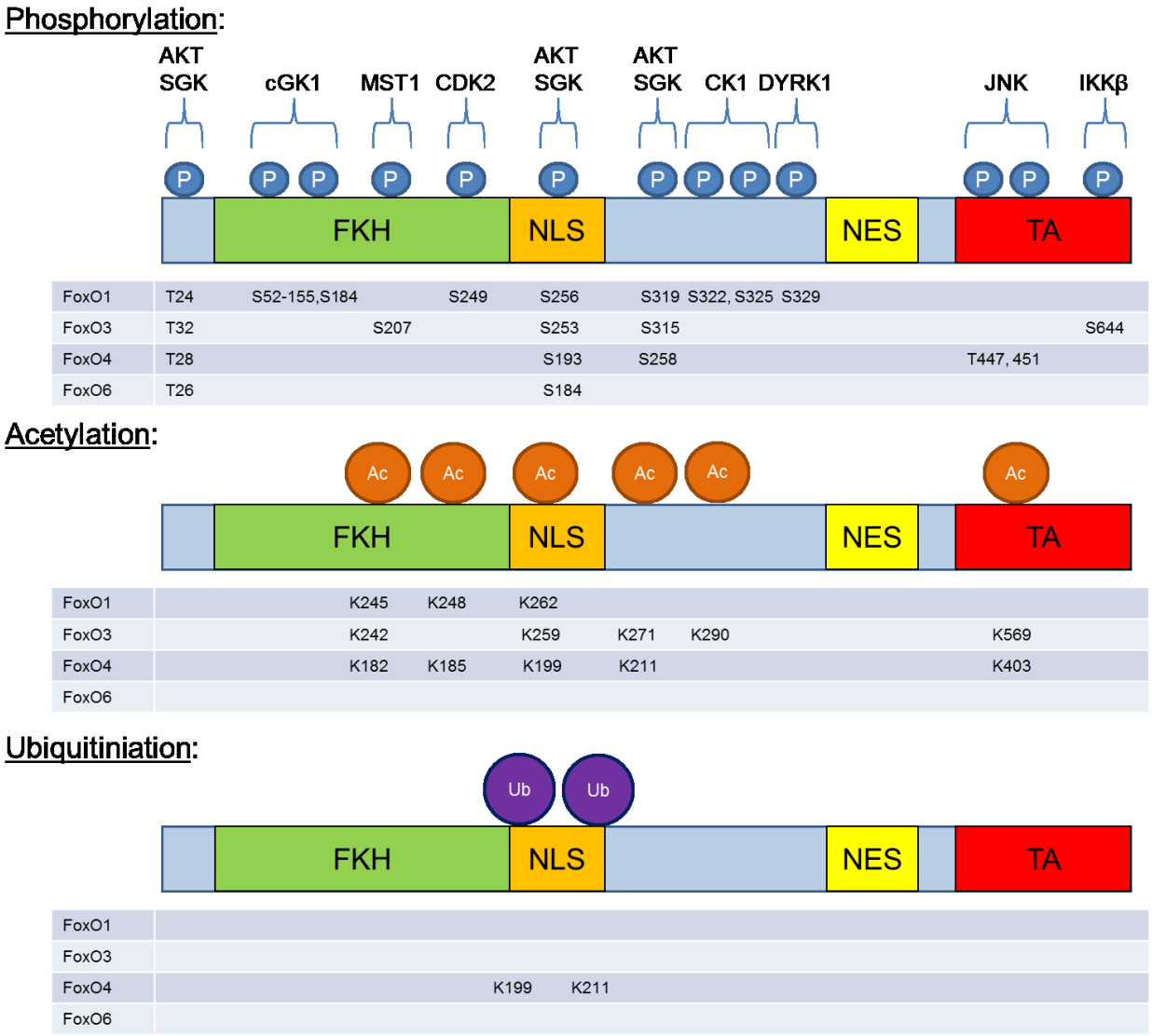

Figure 6: Schematic overview of post translational modification (PTMs) sites of FoxOs
FKH: Forkhead domain; NLS: Nuclear localization sequence; NES: Nuclear export signal;
TA: Transactivation domain; Kinases for specific indicated phosphorylation-sites are indicated above the site.

Beside the mentioned major phosphorylation sites, FoxO proteins also include additional phosphorylation sites like S207 and S644 in FoxO3a. MST1 for instance, phosphorylates FoxO3a at the S207 site and is induced by oxidative stress [28]. As phosphorylation at specific sites determine activation, acetylation on the same sites, show a controversy according to their effect on the FoxO activity [29]. This controversy of PTMs to specific sites led to the suggestion that cell-cycle related genes are upregulated by acetylation whereas pro-apoptotic genes are down-regulated [30]. Arginine 248 and arginine 250 - methylation of FoxO1 on the other hand has been reported to interfere with the AKT mediated phosphorylation resulting in decreased transcriptional activity [31]. The complexity of these modifications and their effects on the functionality of Forkhead Box Family O members is not completely understood yet and will require further work.

1.8 A strong association between FoxO3a activity and arthritis

Perturbations in FoxOs have been linked to RA pathogenesis. Thus, decreaed levels of FoxO1 in PBMC were detected in RA patients when compared to healthy individuals [56]. In contrast, FoxO3a levels were increased in the peripheral blood from patients with RA when compared with controls [56]. It was also previously shown that FoxO1, FoxO3a, and FoxO4, are expressed and phosphorylated in RA synovial tissue. More strikingly, a single-nucleotide polymorphism (SNP) in FoxO3a (rs12212067; T>G) was recently shown to associate with RA disease activity and radiographic progression. In more detail, Lee et al. used genome-wide association studies to uncover a noncoding polymorphism in FoxO3a (rs12212067: T>G) which impacts the inflammatory response in macrophages [36]. Macrophages with the minor Genotype (G), which was assocaited with a milder disease, produced less pro-inflammatroy cytokines (e.g. TNFα), but more anti-inflammatory mediators, such as IL-10. Correspondingly, collagen-induced arthritis (CIA), which is a well established modle to study RA, was pronounced in mice with loss-of-Foxo3 function[35,36]. By contrast, FoxO3a deficiency ameliorated symptoms in the K/BxN serum transfer arthritis model [55]. In this arthritis mouse model, FoxO3a was shown to support neutrophil survival during the course of inflammation [55]. This conflicting data suggest that FoxO3a may exhibit cell-type specific pro- or anti-inflammatory role. Nonetheless these studies indicate that FoxO3a mediates critical fate decisions in synovial inflammation. To unravel its potential pathogenic involvement in RA we explore the biological consequences of FoxO3a regulation in FLS.

1.9 Aim of the thesis

The role of FoxO3a in RA-FLS remains elusive. Therefore, the aim of this study is to gain a better understanding of how FoxO3a regulates the inflammatory response in FLS. Since TNFα, the apex of proinflammatory cytokines in RA, is a major regulator of FLS, this thesis focuses on FoxO3a´s contribution to TNFα-driven FLS activation. Specifically, we investigate the role of FoxO3a in regulating inflammatory gene expression.

2 Experiments and Results

2.1 Phosphorylation of FoxO3a by TNFα in RA-FLS

2.1.1 TNFα stimulation of RA-FLS results in phosphorylation of FoxO3a

It was previously shown that the PI3K-AKT pathway, which regulates FoxO3a activity, is activated in the rheumatoid synovium [30]. Moreover, it was shown that TNFα can activate the PI3K-AKT signalling circuit in FLS. We therefore hypothesized that TNFα may promote FoxO3a phosphorylation via the PI3K-AKT pathway in RA-FLS. To test this assumption we isolated RA-FLS and treated these cells with TNFα [10ng/ml] for various time points. After stimulation FLS were harvested and lysed with RIPA buffer. Western blots indeed revealed that TNFα promoted T32 phosphorylation of FoxO3a.

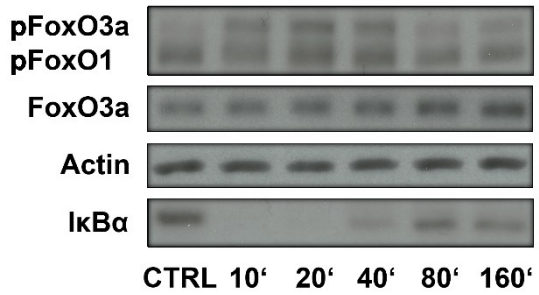

Figure 7: TNFα dependent phosphorylation of FoxO3a in RA-FLS.
RA-FLS were stimulated with TNFα [10 ng/ml] for indicated time points. Representative blots of 3 independent experiments with FLS cell lines from 3 different donors are shown.

2.1.2 TNFα-induced FoxO3a phosphorylation depends on AKT

Our initial experiments revealed that TNFα induces phosphorylation of FoxO3a in RA-FLS. We next asked whether AKT is responsible for TNFα-induced FoxO3a phosphorylation. Therefore, RA-FLS were stimulated with TNFα [10ng/ml] in the presence or absence of the well-known allosteric AKT inhibitor MK2206 [46].

MK2206, indeed, prevented the TNFα-induced phosphorylation of AKT and FoxO3a, demonstrating that TNFα activates AKT to subsequently phosphorylate FoxO3a in RA-FLS.

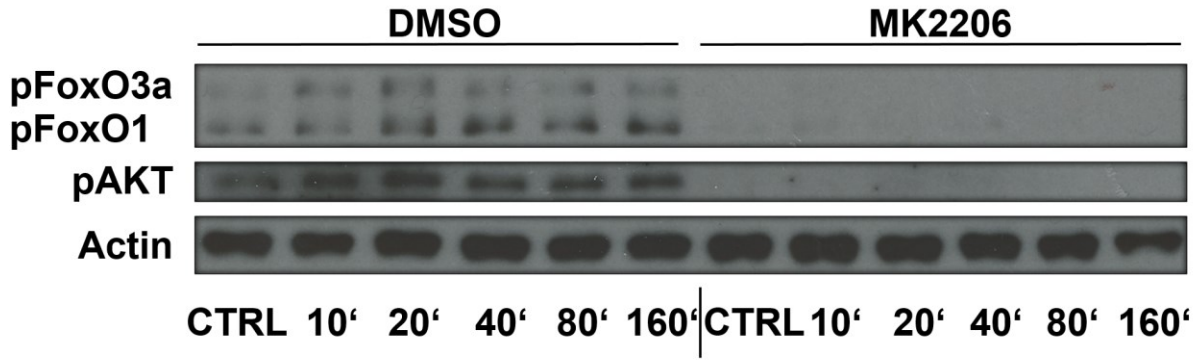

Figure 8: Inhibition of FoxO3a phosphorylation by the AKT-Inhibitor MK2206
RA-FLS were stimulated with TNFα (10 ng/ml) in the presence or absence of the AKT-inhibitor MK2206 (1μM) for indicated time points. Representative blots of 3 independent experiments with FLS cell lines

2.2 Translocation of FoxO3a due to stimulation of TNFα

2.2.1 Immunofluorescence staining of FoxO3a

It has been reported several times that phosphorylation of FoxOs by AKT results in FoxO nuclear exclusion [22, 25, 26]. To examine whether TNFα-induced phosphorylation of FoxO3a also results in FoxO3a cytoplasmic migration we next performed immunofluorescence staining and imaging of TNFα treated and untreated RA-FLS. TNFα stimulation indeed resulted in nuclear exclusion of FoxO3a. The nuclear-to-cytoplasm shuttling seems to occur in a 2-stage pattern. 3h after TNFα stimulation, FoxO3a undergoes a nuclear recovery followed by a second cytoplasmic translocation.

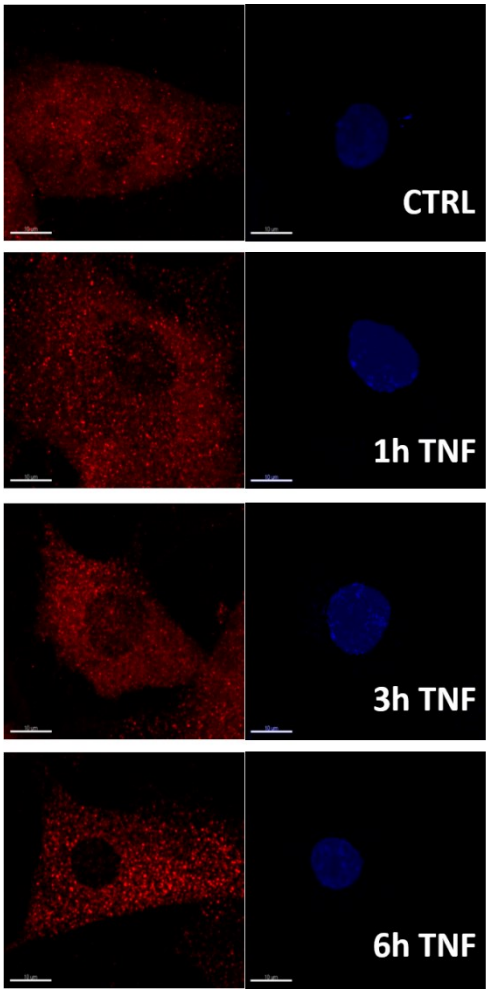

Figure 9: TNFα induced shuttling of FoxO3a in FLS
Cells were seeded on cover slides, starved and stimulated with TNFα [10ng/ml] for indicated time points. After fixation and permeabilization, FLS were stained in an indirect assay for FoxO3a (red) and counter-stained with DAPI for nuclei detection. Pictures were taken with an inverse confocal microscope. Representative Images of 1 patient are shown.

2.2.2 Automated IF microscopy and quantification of FoxO3a translocation

In order to quantify the observed translocation of FoxO3a in TNFα stimulated FLS, we performed an automated microscopy single cells immunofluorescence analysis in 96-Well plates. Foxo3 was stained in an indirect assay. DAPI counter-staining was used for determination of the nuclear area. The perinuclear area was defined as an 11.4µm extended ring. The mean of FoxO3a signal Intensity in the nuclear vs. perinuclear area was used for calculation of the nuclear translocation.

Automated IF could repeat and quantify the results of the previous experiment. After TNFα stimulation, nuclear FoxO3a content are decreased, but recovered within 80 minutes after TNFα stimulation. The recovery is followed by a second wave of nuclear exclusion which is sustaining through the experimental timeframe.

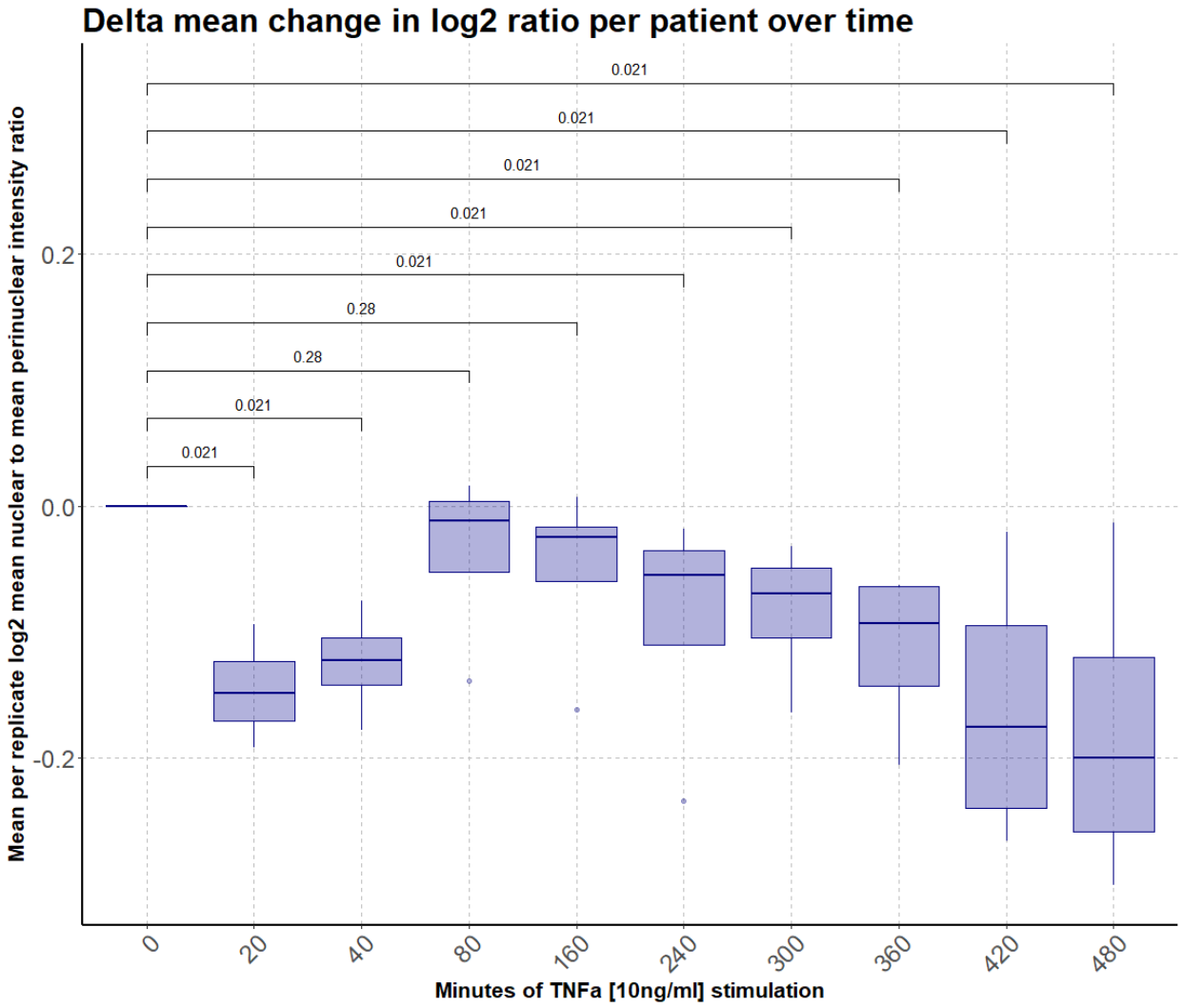

Figure 10: Immunofluorescence of nuclear Translocation via automated high throughput, high content automated microscopy
Cells were seeded in 96-Well plate. After starvation, FLS were stimulated with TNFα [10ng/ml] for indicated time points.Afterwards cells were permeabilized and fixed. Single cell selection and analysis was performed to avoid overlapping of the FLS cell bodies by a neighbourhood distance of 14µm. Nuclear Area was determined by DAPI, perinuclear area was determined as 11.4µm wide ring around the nuclear area. Mean Intensity of FoxO3a in the selected areas was used for further calculation. FLS from 4 different patients were used. Data were clustered and used for statistical analysis. Bonferroni-corrected multiple T-test were used.

2.3 Identification of TNFα dependent FoxO3a regulated genes

To decipher cellular processes that are regulated by FoxO3a, we performed siRNA mediated depletion of FoxO3a in RA-FLS under TNFα treatment. Changes in the global transcriptome were measured by RNA sequencing. TNFα regulated genes were characterized by comparison of unstimulated vs. 6h TNFα stimulated FLS treated with non-coding siRNA. As expected, TNFα induced an increased expression of well-known proinflammatory cytokines (e.g. IL6), proteolytic enzymes (e.g. MMP3) and inflammatory transcription factors (e.g.IRF3) in RA-FLS. Overall, 264 genes were downregulated and 770 genes were upregulated (fold change >2, p-value<0.05). Those 770 upregulated genes were further analysed according to siRNA mediated FoxO3a silencing. 34 genes revealed to positively and 28 genes were negatively regulated by FoxO3a. Numerous TNFα-regulated genes that were affected by the FoxO3a knockdown, such as CXCL9, CXCL10, CXCL11 or TNFSF13B, are known to contribute to the progression of the synovial inflammation in RA. Gene ontology (GO) term analysis of these genes revealed functions important for the inflammation in the synovium, such as cell proliferation, chemotaxis or immune response. To summarize these results, FoxO3a showed a specific regulatory function in the expression of TNFα-induced pro-inflammatory cytokines and chemokines in RA-FLS.

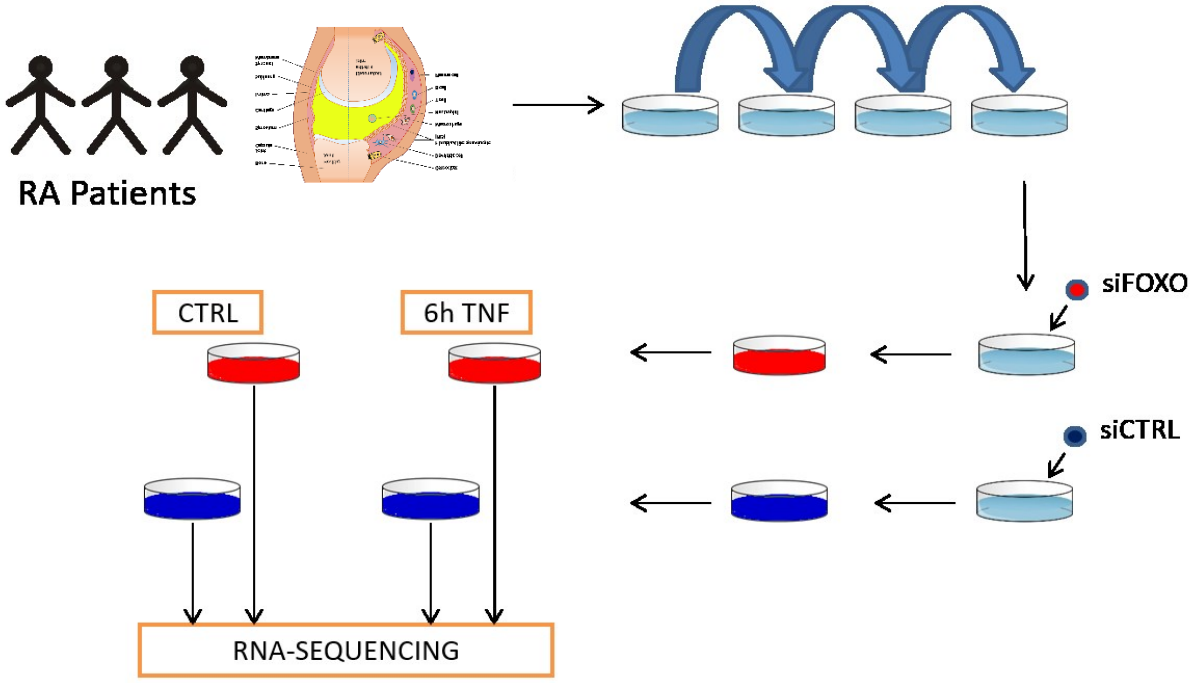

Figure 11: Scheme of RNA-sequencing of FLS
Synovial tissue of RA patients was digested via collagenase II and reseeded until passage IV. Cells were transfected with a lipofectamin-siRNA complex. FLS were seeded, starved and stimulated with TNFα [10ng/ml] for 6h. FLS were lysed, RNA was isolated and analysed by RNA-sequencing.

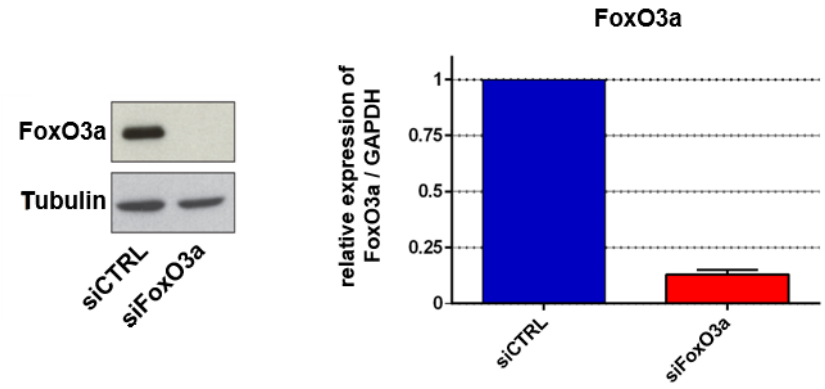

Figure 12: Control of FoxO3a knockdown efficiency
FoxO3a targeting SiRNA-pool transfected RA-FLS were reseeded in a 6-well plate, starved and stimulated for 6h with TNFα [10ng/ml]. Knockdown efficiency was determined by western blotting or RT-qPCR (n=3)

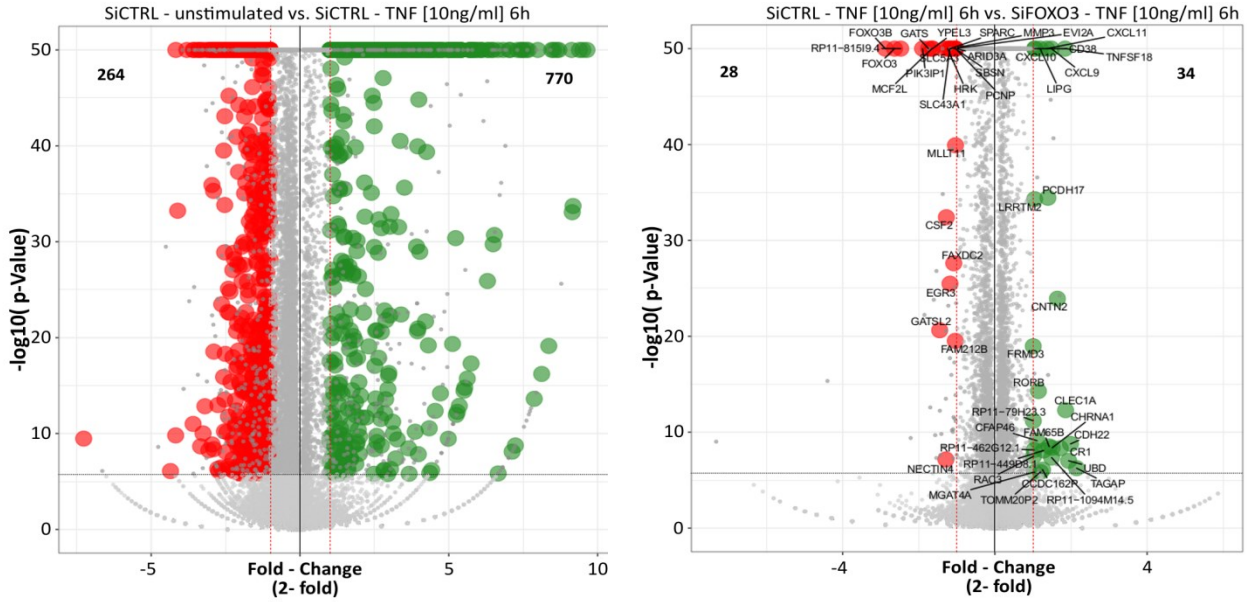

Figure 13: RNA-Sequencing – TNFα induced genes / FoxO regulated genes
RNA Sequencing data of 3 patients were analysed according to the TNFα induction of genes (p-value < 0.05, fold-change > 2). Unstimulated vs. 6h TNFα [10ng/ml] SiCTRL-knockdowned FLS were used to identify TNFα-regulated genes. 770 genes were identified as TNFα-positive regulated and 264 genes were identified as TNFα-negative regulated. TNFα up-regulated genes were further analysed in unstimulated vs. 6h TNFα FoxO3a-knock downed FLS. Resulting in 34 genes identified as FoxO3a positive regulated as well as 28 FoxO3anegative regulated genes.

2.4 Validation of RNA-Sequencing data

RNA Sequencing data was confirmed by detection of TNFα-inducible inflammatory mediators by ELISA. Consistent with the transcriptomic data, an increased translation of CXCL10 in TNFα treated, siRNA mediated FoxO3a knock downed RA-FLS could be observed. Furthermore, a decrease in the expression of MMP3 could be detected. Similar to the RNA-sequencing data, a siRNA mediated FoxO3a knockdown did not alter the expression of IL6 or IL8, which indeed confirmed the specific regulation of TNFα-induced genes in RA-FLS.

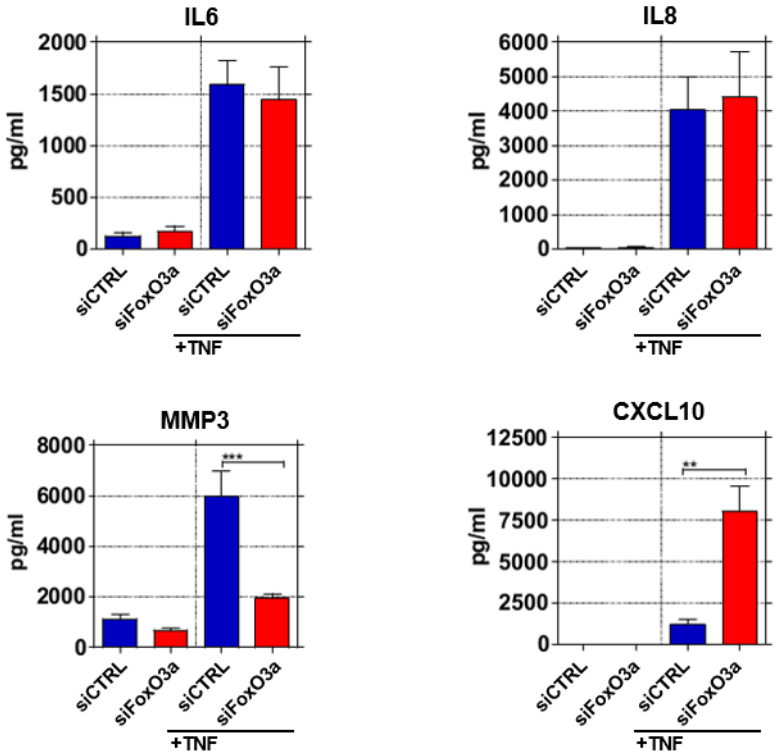

Figure 14: Validation of RNA-sequencing data on protein level
FLS transfected with non-targeting siRNA or FoxO3a targeting siRNA pools were seeded in 6-well plates, serum-starved and stimulated for 24h with TNFα [10ng/ml]. Supernatants were used to perform ELISA assays. FLSfrom3 different donors were analysed.

2.5 Gene annotation of TNFα dependent FoxO3a regulated genes

The listed TNFα dependent FoxO3a up- and down-regulated genes have been further analysed in concerns of their GO Terms in order to categorize the role of FoxO3a in the biological process of RA. This result underlines the dual role of FoxO3a in RA FLS as stress response mediator as well as organizer for the adaptive immune system by inducing and regulating cell-migration through chemotaxis.

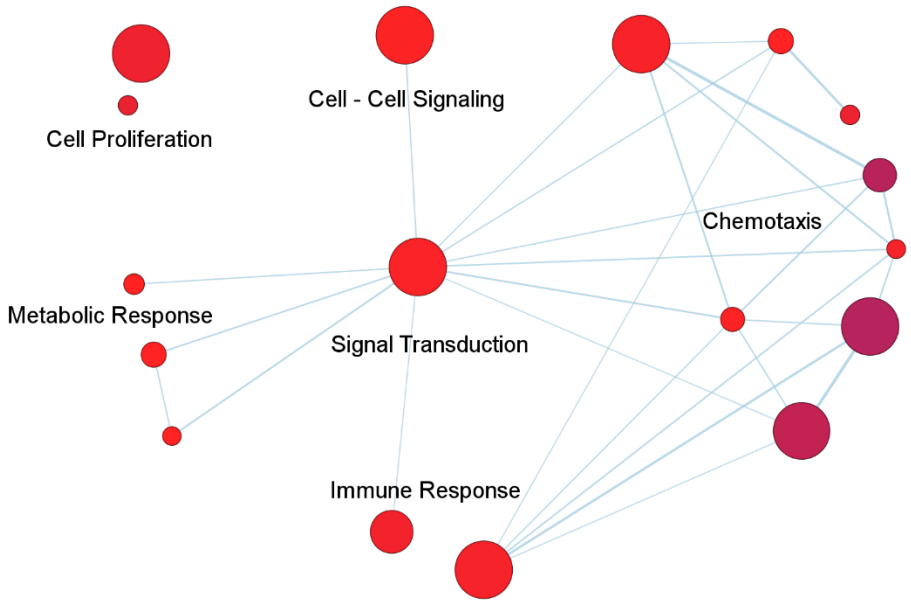

Figure 15: Gene annotation of TNFα dependent, FoxO3a regulated genes
TNFα induced (FC>2, p-Value<0.05) FoxO3a regulated (FC>2, p-Value<0.05) protein coding genes have been annotated to their Biological process using DAVID V6.7. Gene Ontology Terms have been clustered and mapped according to their response (Dot-size represents the number of genes included in the term, colour gradient indicates the p-Value: (red=0, blue=0.2).

3 Materials and Methods:

3.1 Cell-culture:

With approval by the ethics committee synovial tissues from patients fulfilling the ACR/EULAR classification criteria for RA were obtained as discarded specimens following synovectomy. RA-FLS were isolated by tissue digestion with 1mg/ml collagenase type IV for 1h in 37°C by gently rocking in HEPES-buffered saline (HBS) solution (20mmol/L) HEPES, 137 mmol/L NaCl and 3mmol/l KCl, pH=7.4) The cell suspension was passed through a 70µm cell strainer and placed in tissue culture flasks150. FLS were cultured in 25ml Dulbecco's modified eagle medium (DMEM) complete in T125 Flasks with weekly media-exchange. Cells were split at 80% confluence and used from passage 3 to passage 10 for further experiments. Environmental conditions for cell culture were 37°C with 5% CO_2.

3.1.1 Reagents

3.1.1.1 **DMEM complete**

Dulbecco's modified eagle medium (DMEM)	450ml
Fetal calf serum (FCS)	50ml
Non-essential amino acids (NEAA)	5ml
Penicillin/ Streptomycin (Pen/Strep)	5ml

3.1.1.2 **Starving Medium**

DMEM	450ml
Bovine Serum Albumin (BSA)	562mg (=0.125%)
NEAA	5ml
Pen/Strep	5ml

3.1.1.3 **Transfection Medium**

DMEM	450ml
FCS	50ml
NEAA	5ml

3.1.1.4 **DMEM:**

GIBCO – DMEM 500ml, #42430-25

3.1.1.5 **FCS:**

Thermo Fisher – High Clone Fetal Calf serum, #16140071

3.1.1.6 **NEAA:**

GIBCO – NEAA, #11140-035

3.1.1.7 **Pen/Strep:**

GIBCO – Pen Strep 100ml, #15140-122

3.1.1.8 **Trypsin EDTA 10x**

GIBCO – 0.5% Trypsin EDTA 10x 100ml, #15400-054

3.1.1.9 **BSA:**

Sigma Aldrich – Bovine Serum Albumin, #A4503-100G

3.1.1.10 **Trypanblue**

Filtered Trypanblue

3.1.1.11 **Trypanblue:**

Sigma Aldrich – Trypanblue Solution 4% 100ml, #T8154

3.1.1.12 **Collagen Type II**

Sigma Aldrich – Collagen Type II #C9301-5MG

3.1.2 Material

3.1.2.1 **6-Well plates**

TPP – 6-Well tissue culture plates, #92006

3.1.2.2 **24-Well plates**

Costar – 24-Well plates for tissue culture, #3524

3.1.2.3 **96-Well plates (for ELISA)**

TPP – 96-Well tissue culture plates, #92096

3.1.2.4 **96-Well plates (for ELISA Assay)**

Sigma – Nunc-Immuno MicroWell 96 well solid plates, #M9410

3.2 Passaging:

Culture medium was aspirated and adherent cells were washed once with PBS. 3ml of Trypsin was added and flasks were incubated for 8 minutes at 37°C. Trypsinization was stopped by the addition of 7ml DMEM complete. Cells were centrifuged at 400g for 8min at room temperature. The supernatant was aspirated and the pellet resuspended in 10ml DMEM complete. Cells were counted via a Neubauer chamber, set to a proper concentration and seeded according to their density.

3.2.1 Cell counting:

10µl of the cell suspension was mixed with 10µl Trypanblue and loaded onto the Neubauer chamber. Cells were counted and concentration was calculated via the following formula:

$$c_{Cells} = \frac{n_{Cells}}{4} * f_{dilution} * 10^4$$

c_{Cells} : Cell density [Cells/ml]
n_{Cells} : Number of cells in all 4 areas [Cells]
$f_{dilution}$: Dilution factor [1]

3.2.2 Seeding:

After cell counting, cells were diluted with DMEM complete to following concentrations and homogenously seeded overnight at 37°C, 5% CO_2. Cells were controlled next day under the microscope for homogenous distribution, adherence and fibroblast like cell formation.

3.2.3 Cell density:

Plate	*Experimental Design*	*Cell density*
6-Well plate	Western blot	1,5*10^5 cells/ Well
	Si-mediated Knockdown	1,5*10^5 cells/ Well
	Cells for ELISA	1,5*10^5 cells/ Well
	RNA-Samples for qPCR	1,5*10^5 cells/ Well
24- Well plate	Immunofluorescence (cover slides)	7.5*10^3 cells/ Well
96- Well plate	Immunofluorescence	7*10^3 cells/ Well
	ELISA Assay	

3.2.4 Inhibitors and Stimulation:

Cells were treated with Inhibitors and Stimuli in following concentrations:

Substance	*Type*	*Solvent*	*Concentration*	*Storage condition*
rhTNFα	Stimulant	PBS	10ng/ml	-20°C
MK2206	Inhibitor	DMSO	1µM	-20°C

3.2.4.1 **TNFα**

R&D Systems – Recombinant Human TNFα, #210-TA-020

3.2.4.2 **MK2206**

Selleckchem – MK-2206 2HCl, #S1078

3.3 Protein purification:

3.3.1 Reagents:

3.3.1.1 **RIPA complete (for 1ml)**

RIPA Buffer	988µl
HALT Phosphatase-Inhibitor	10µl
Protease Inhibitor-mix	1µl
Benzonase	1µl

3.3.1.2 **RIPA Buffer**

Thermo Fisher – RIPA Lysis and Extraction Buffer, #89900

3.3.1.3 **HALT Phosphatase Inhibitor**

Thermo Fisher – Halt Phosphatase Inhibitor Cocktail, #78420

3.3.1.4 **Protease Inhibitor mix**

Sigma Aldrich – Protease Inhibitor Cocktail, #P8340

3.3.1.5 **Benzonase**

Millipore – Benzonase Nuclease 250U/µl, #2698486

3.3.1.6 **Lowry Measurement (per Measurement)**

Lowry solution A	98µl
Lowry solution B	800µl
Lowry solution S	2µl

3.3.1.7 **Lowry solution A**

BIO RAD – DC Protein Assay Reagent A, #500-0113

3.3.1.8 **Lowry solution B**

BIO RAD – DC Protein Assay Reagent B, #500-0114

3.3.1.9 **Lowry solution S**

BIO RAD – DC Protein Assay Reagent S, # 500-0115

3.3.1.10 **BSA-Standard**

Sigma Aldrich - BSA for protein quantification, #1076192

3.3.2 Devices:

3.3.2.1 **Centrifuge**

MIKRO 200R – Hettich Centrifuges - Bartelt

3.3.2.2 **Photometer**

BioPhotometer - Eppendorf

3.3.3 FLS Harvesting for western blot:

After the incubation, the culture medium was aspirated gently from the 6-Well plate. Cells were gently rinsed 2x with PBS (4°C). Rinsed cells were lysed with 100µl RIPA complete per well on 4°C. The cells were scraped and transferred into an Eppendorf reaction tubes (“eppi”).

3.3.4 Purification of western blot samples:

The protein solution was incubated on 4°C for 30 minutes with 15s vortex-steps every 10 minutes. After Incubation cells were centrifuged in a microcentrifuge for 15min at 4°C for 15.000RPM (=16.000g). Supernatants were transferred into a new eppi. The pellet containing the cell debris was discarded.

3.3.5 Determining concentration (Lowry):

To determine the protein concentration, the BIORAD-DC protein assay was used. For each sample, 2µl of solution S and 98µl of solution A were mixed and named solution A’. 10µl of Sample were added to 100µl of solution A’ and mixed properly via pipetting several times. After the addition of 800µl of solution B, the Sample-A’-B Solution was vortexed and transferred into the cuvettes where the complex was formed and measured after 15 minutes. For the standard, 1µl, 2.5µl, 5µl, 10µl and 15µl of the BSA Standard was used instead of the 10µl Sample. The complex was measured via photometer at 595nm. The concentration was calculated according to a linear regression of the Absorbance/concentration ratio.

3.4 Western-blotting (Wet-blot)

3.4.1 Reagents:

3.4.1.1 **10x Base Buffer**

Glycine	144g
Tris-Base	30,3g
ddH_2O	ad 1000ml
Stored at Room temperature	

3.4.1.2 **10% SDS Solution**

SDS	10g
ddH2O	ad 100ml
Stored at Room temperature	

3.4.1.3 **Running-Buffer**

10x Base Buffer	100ml
10% SDS Solution	10ml
ddH_2O	ad 1000ml
Stored at 4°C – 5x reusable	

3.4.1.4 **Blotting-Buffer**

10x Base Buffer	100ml
Methanol	200ml
ddH_2O	ad 1000ml
Stored at 4°C – 5x reusable	

3.4.1.5 **10x TBS**

Tris-Base	24g
NaCl	88g
ddH_2O	ad 900ml
Set pH = 7.6 with HCl/NaOH	
ddH_2O	ad 1000ml
Stored at Room temperature	

3.4.1.6 **Glycin**

Applychem – Glycine Molecular Biology Grade, #A1067, 1000

3.4.1.7 **Tris-Base**

Merck – Tris(hydroxymethyl)aminomethan, #8382G012 505

3.4.1.8 **SDS**

CALBIOCHEM – Sodium n-Dodecyl Sulphate Molecular Biology Grade, #428023

3.4.1.9 **Methanol**

Fisher Chemical – Methanol Analytical reagent grade, #M/4000/17

3.4.1.10 **NaCl**

Merck – NaCl, #106404100

3.4.1.11 **TBST**

10x TBS	100ml
Tween 20	1ml
ddH_2O	ad 1000ml

Stored at Room temperature

3.4.1.12 **Blocking Buffer**

Milk powder / BSA	5g
TBST	ad 100ml

Stored at 4°C for 1 week

3.4.1.13 **Tween 20**

Merck – Tween 20, #8.22184.0500

3.4.1.14 **Milk powder**

Roth – Powdered Milk, #T145.1

3.4.1.15 **BSA**

Sigma Aldrich – Bovine Serum Albumin, #A8022-100G

3.4.1.16 **5x Laemmli Buffer**

Laemmli Sample Buffer (4x)	875µl
B-Mercaptoethanol	125µl

Aliquoted and stored at -20°C

3.4.1.17 **4x Laemmli Sample Buffer**

BIO RAD – 4x Laemmli Sample Buffer, #161-0747

3.4.1.18 **β-Mercaptoethanol**

Sigma-Aldrich – 2-Mercaptoethanol, #M-3148

3.4.1.19 **Protein-Ladder**

Thermo Fisher - Page Ruler Prestained Protein ladder, #26616x4

3.4.1.20 **Stacking-PAA-gel (for 1 gel, 1.5mm)**

ddH_2O	3.73ml
Acrylamide 30%	836µl
Stacking gel buffer	1.575ml
SDS 10%	62.5µl
APS 10%	62.5µl
Temed	12.5µl

3.4.1.21 **Staking Gel Buffer**

BIORAD – Stacking Gel Buffer, #161-0799

3.4.1.22 **Separating-PAA-gel (for 1 gel, 1.5mm)**

ddH_2O	4 ml
Acrylamide 30%	3.35ml
Separating gel buffer	2.5ml
SDS 10%	100µl
APS 10%	50µl
Temed	10µl

3.4.1.23 **Separating Gel Buffer**

BIORAD – Resolving Gel Buffer, #161-0798

3.4.1.24 **Acrylamide-Bisacrylamide Solution**

BIORAD – 30% Acrylamide/Bis Solution 29:1, +161-0156

3.4.1.25 **10% Ammonium persulfate (APS)**

Sigma-Aldrich – APS, #A3678

3.4.1.26 **TEMED**

BIORAD- TEMED, #161-0800

3.4.1.27 **SDS**

Calbiochem – SDS, #428023

3.4.1.28 **Isopropanol**

Merck – 2-Propanol, #603-117-00-0

3.4.1.29 **Wattman Paper**

BIOREAD – Mini Trans-Blot Filter paper, #1703932

3.4.1.30 **Nitrocellulose Membrane**

STRATAGENE – Nitrocellulose Membrane, #420108

3.4.1.31 **Primary antibodies**

FoxO3a	Cell Signalling – FoxO3a (D19A7) Rabbit mAb, #12829
pFoxO3a	Cell Signalling – Phospho-FoxO3a (Thr32) mAb, #2599
AKT	Cell Signalling – AKT Antibody mAb, #9272
pAKT	Merck – Phospho-AKT (Thr308) Antibody, #07-1398
IκBα	Cell Signalling – IκBα (L35A5) Mouse mAb, #4814
Actin	Sigma Aldrich - Anti-Actin antibody produced in rabbit pAb, #A2066

3.4.1.32 **Secondary antibodies**

Rabbit-HRP linked	Cell Signalling – Anti-rabbit IgG, HRP-linked Antibody, #7074
Mouse-HRP linked	Sigma Aldrich – Anti-mouse IgG, HRP-linked Antibody, #SAB3701023

3.4.1.33 **ECL substrates (for 1 membrane)**

Peroxide agent	500µl
Luminol/enhancer	500µl

3.4.1.34 **ECL substrate**

BIORAD – Clarity ECL Western substrate, #1705061

3.4.1.35 **Film Developer**

AGFA – CURIX60

3.4.1.36 **Radio film**

GE Healthcare – Amershame Hyperfilm ECL, #28906837

3.4.1.37 **Stripping solution**

Merck Re-Blot Plus Strong Solution (10x)	1ml
ddH_2O	9ml

3.4.2 Devices:

3.4.2.1 **Electrophoresis-chambers**

BIORAD – Mini-PROTEAN® Tetra Cell Systems

3.4.2.2 **Thermocycler**

Eppendorf – ThermoMixerC

3.4.2.3 **Power Supply**

BIORAD – Power Pac HC

3.4.3 Preparing the gels and buffers

Buffers were prepared 24h before usage and stored at 4°C. Pre-gel solutions were prepared without addition of APS and Temed. After addition of 50 µl Ammonium persulfate and 10µl TEMED to the Separating-PAA-gel-solution the solution was quickly homogenized via multiple pipetting steps and 5ml were pipetted into the 1.5mm PAA-Gel-chamber. Additional 700µl of Isopropanol were used as an overlay during the polymerization of the PAA-Gel. After 45minutes the Isopropanol was carefully removed and 62.5µl of Ammonium persulfate as well as 12.5µl of TEMED were added to the Stacking-PAA-gel-solution. The solution was quickly homogenized and 2.5ml of the stacking gel were pipetted onto the separating gel. The chomps were inserted into the stacking gel before the polymerization occurs. After 20 minutes the gels were put into wet towels and stored at 4°C in closed bags to avoid drying of the gels for up to 1 Week.

3.4.4 Preparing samples for PAA-Gel electrophoresis

Protein samples were aliquoted according to their protein-concentration. 70µl of the sample with the lowest protein concentration was mixed with 20µl of 5x Laemmli Buffer. The other samples were adjusted with RIPA complete to a volume of 70µl and mixed with 20µl of 5x Laemmli-Buffer. The prepared samples were denatured at 95°C for 5 min and stored at -20°C.

3.4.5 SDS PAGE

Gels were put into the Mini-PROTEAN® Tetra Cell system, the system was filled with Running-Buffer according to the number of gels. Chomps were carefully removed and the slots were flushed with running-buffer and filled with 15µl of samples or 2µl of the prestained page-ruler marker. Slots without marker or sample were filled with 15µl of a RIPA complete/ 5x Laemmli Buffer mixture with the same Laemmli concentration as the samples. Gels were run at 120V for 15min and at 100V for 45 minutes.

3.4.6 Blotting

Run gels were transferred into the blotting device with blotting buffer soaked Wattman paper mantling the gel and the nitrocellulose membrane. Air bubbles were gently removed and membranes were blotted at 100V for 1h.

3.4.7 Poncou S-staining

The membrane was stained for 1 minute with Poncou S and destained with ddH_2O to verify a correct and equal distributed blotting without air bubbles.

3.4.8 Blocking and primary Antibody

The membrane was blocked in Blocking buffer for 1h at room temperature on the shaker. The membrane was cut with a scalpel according to the primary antibodies target molecular weight. Primary antibodies were diluted in blocking buffer (BSA/milk powder, according to the manufacturer's protocol).The membranes were put into a 50ml Falcon tube without overlapping of the membrane with 5ml primary antibody solution and mixed at a roll mixer at 4°C over night.

3.4.9 Secondary Antibody

The membranes were washed 3x for 10 minutes in TBST. Secondary antibody was diluted 1:1000 in Blocking buffer (milk powder).The membrane was incubated for 1h at room temperature. After secondary antibody membranes were washed 3x 10 minutes at room temperature with TBST.

3.4.10 Detection

The ECL substrates were mixed and placed as a droplet on a clean plastic-foil. The membrane was placed upside-down onto the droplet and carefully moved to cover the whole area equally with the substrate for 1 minute. After ECL Incubation, the membrane was placed in a cassette in a clean plastic foil with carefully avoidance of air bubbles. The cassette and films were taken into a dark room and the films were developed after 1 to 45 minutes exposure.

3.4.11 Stripping and reblotting with primary antibody

After the development of the film the membrane was washed again 3 times with TBST to get rid of the ECL solution and stripped with the stripping solution for 10 minutes. The stripping was stopped by washing 1x with blocking buffer for 1 minute and the membrane was blocked for 1h at room temperature with blocking buffer. The blocked membrane was then incubated with 5ml the primary antibody solution in a 50ml falcon tube over night at 4°C at the roll mixer.

3.5 Si mediated Knockdown

3.5.1 Reagents

3.5.1.1 **SiRNA's**

siCTRL	Dharmacon – ON-TARGET plus Non-targeting Control Pool, 20nmol #D-001810-10-20
siFoxO3a	Dharmacon – ON-TARGET plus Human FoxO3a (2309) siRNA – SMART pool, 5nmol #L-003007-00-0005

3.5.1.1.1 Si-RNA mediated Knockdown-solutions

3.5.1.2 **SiRNA-solution**

SiRNA [20µM]	5µl
Optimem	245µl

3.5.1.3 **Lipofectamine-solution**

Lipofectamine	5µl
Optimem	245µl

3.5.1.4 **Optimem**

GIBCO – OPTIMEM, #31985-062

3.5.1.5 **Lipofectamine**

Thermo Fisher – Lipofectamine LTX Reagent with Plus Reagent 1ml, #1538100

3.5.2 Seeding and Transfection

150.000 cells per well were seeded in a 6-well plate in 1.5ml transfection media overnight. Next day, 250µl of siRNA-solution as well as 250µl of the Lipofectamin-solution were mixed together and incubated for 20 minutes at room temperature to form the transfection mix. The cells were incubated with 500µl of the transfection mix for 72h. Cells were trypsinized, counted and reseeded according to the conditions of the experiment. Knockdown efficiency was determined via western blot or RT-qPCR.

3.6 RNA purification

3.6.1 Reagents

3.6.1.1 **Qiagen RNeasy Kit**

RLT Buffer, #79216
RW1 Buffer, #1053394
RPE Buffer, #1018013

3.6.1.2 **Qiagen RNeasy Kit**

Qiagen – Rneasy kit, #74104

3.6.2 Devices

3.6.2.1 **Micro centrifuge**

Bartelt – MIKRO 200R

3.6.2.2 **Nanodrop**

Thermo Fisher – NANODROP LITE

3.6.3 RNA Harvesting

Post stimulation, the medium was aspirated gently from the 6-Well plate. Cells were gently rinsed 2x with PBS (4°C). After aspiration of the PBS the cells were treated with 350µl RLT buffer per Well on room temperature. The cells were scraped and transferred into an RNase free eppi. The lysate was centrifuged for 3 minutes at 15kRPM at room temperature. The supernatant was carefully transferred into a new eppi. 350µl of 70% ethanol was added to the lysate and mixed by pipetting. The mix was immediately transferred onto an RNeasy Mini spin column placed in a 2ml collection tube. The mix was centrifuged for 1 minute at 15kRPM at room temperature and the flow-through was discarded. 700µl of RW1 Buffer were loaded onto the column, centrifuged again for 1 minute at 15kRPM at room temperature and the flow-through was discarded. The RPE buffer was completed according to the manufacturer's protocol and used to wash the column for 2x by centrifugation for 15 kRPM, 2 minutes at room temperature. After washing the column was placed in a new autoclaved RNase free eppi, loaded with 30µl RNase free ddH_2O and centrifuged for 2 minutes at 15kRPM at room temperature. RNA samples were stored immediately at -80°C.

3.6.4 Determining concentration

The nanodrop was used to determine the RNA concentration. RNase free ddH_2O was used to clean the nanodrop and blank. The ratio between 260nm/280nm was used to determine the purity. Samples with a ratio outside of 1.9-2.15 were purified by usage of the RNA purification kit.

3.6.5 RNA purification

The RNA sample was adjusted to a total volume of 100µl with RNase free ddH_2O and mixed with 350µl RLT buffer. The Samples were precipitated with 250µl Ethanol and immediately loaded onto a new column. The column was centrifuged for 1 minutes at 15 kRPM at room temperature. The flow-through was discarded and 500µl of RPE buffer were added and the column was centrifuged again for 1 minute at 15kRPM.The flow-through was discarded again and the column was loaded with 500µl of 80% Ethanol. After centrifugation for 3 minutes at 15kRPM the flow-through was discarded again, and the column was placed in a new eppi. After addition of 20µl RNase free ddH_2O the columns were incubated for 1 minute and centrifuged for 3 minutes at 15kRPM. After purification the concentration of the samples were again determined via nanodrop.

3.7 RNA Sequencing

3.7.1 RNA Sequencing

The purified RNA samples of 3 patients were analysed at the CeMM. RNA-seq libraries were prepared with TruSeq stranded mRNA sample preparation kit (Illumina) using Sciclone and Zephyr liquid handling robotics (PerkinElmer). The libraries were sequenced on HiSeq 3000 (Illumina) platform using 50bp single-read chemistry. Sequencing data was interpreted and analysed under support and guidance of the Bioinformatician of the Division for internal medicine III at the general hospital of Vienna.
The raw sequencing data was processed with Illumina2bam to generate unaligned BAM files. Sequence reads were mapped onto the human genome annotation release hg38 (GRCh38) using STAR alinger (V2.5.2b). Reads were counted with the summarizeOverlaps function of Bioconductor package GenomicAlignments (version 1.10.1) on the basis of Ensembl transcript annotation version 89 and DESeq2 (version 1.14.1). Gene expression values (RPKM, reads per kilobase exon per million mapped reads) were calculated with Cufflinks (version 2.2.1). The differential expression between two sample groups were calculated from the raw read counts, with Fisher's exact test for the significance of all samples together (sum of read counts), and edgeR’s (version 3.12.1) dispersion for the variance within biological replicates. The filtering for differentially expressed genes is for p-value (Bonferroni corrected), maximal dispersion of 0.2 and minimal fold-change of 2.

The genes were further analysed according to their TNFα regulation. Therefore the gene expression of the unstimulated vs. 6h TNFα stimulated siCTRL knock downed RNA samples of all patients were analysed. Genes of interest were determined according to their Bonferroni corrected p-Value ($p<0.05$) and fold-change ($fc>2$). TNFα regulated genes were further analysed in order to find FoxO3a regulation. 6h TNFα stimulation of siFoxO3a knock downed FLS vs. 6h TNFα stimulation of siCTRL knock downed FLS was used for analysis expression alterations. TNFα depended FoxO3a regulated genes were identified with a Bonferroni corrected p-value of <0.05 and a fold-change of >2.

3.7.2 Identification of regulation patterns

Identified TNFα dependent, FoxO3a regulated genes were analysed in their biological function to identify regulatory patterns and mode of actions. A gene ontology analysis has been performed using TNFα dependent, FoxO3a regulated genes in a biological process focused GO Term annotation. Genes have been annotated using DAVID [37]. Data have been imaged using Cytoscape v.3.6.1 [38] in a generic analysis asset.

3.8 RT-qPCR

3.8.1 Reagents

3.8.1.1 **Mastermix for qPCR**

ddH_2O	6µl
Cyber green	10µl
Primer forward	1µl
Primer forward	1µl

3.8.1.2 **Mastermix for RT-PCR**

ddH_2O	1.7µl
10x Buffer	2µl
dNTPs	2µl
Oligos	1µl
RT	1µl
RNase OUT	0.3µl

3.8.1.3 **Kits**

Qiagen – Omniscript, #205113

3.8.1.4 **RNase out**

Invitrogen – RNase out 5000U, #10777019

3.8.1.5 **Tag Polymerase**

GIBCO – Tag Polymerase 500U, #18038026

3.8.1.6 **dNTP**

MBI – dNTP Mix 1ml, #R0241

3.8.1.7 **Oligo(dT)**

Sigma – Oligo $(dT)_{23}$, #O4387

3.8.1.8 **DNA Ladder**

GIBCO – 100bp DNA Ladder 50µg, #15628019

3.8.2 Devices

3.8.2.1 **PCR Machine**

Eppendorf – flexid nexus Mastercycler

3.8.2.2 **qPCR Machine**

ROCHE – Light Cycler 480

3.8.3 Reverse Transcription

RNA Samples were diluted with RNase free ddH_2O to a concentration of 42ng/ml in a total volume of 12µl. Aliquoted samples were denaturized for 5 minutes at 65°C. After denaturation, 8µl of mastermix was added to each sample and the reverse transcription was performed at 37°C for 1h. cDNA Samples were stored at -80°C.

3.8.4 qPCR

cDNA Sample concentrations were adjusted to 50 ng/µl and a total volume of 2µl. After addition of 18µl mastermix, the samples were analysed with following temperature program.

Phase		*Temperature*	*Time*	*Cycles*
Initial Denaturing		95°C	10 min	1
PCR	Denaturing	95°C	5 sec	50
	Annealing	65°C	5 sec	
	Extension	72°C	15 sec	
Melting curves	Denaturing	95°C	10 sec	1
	Annealing	65°C	15 sec	
	Acquisition	95°C	Every 5 °C	

3.8.5 Primer List

Gene Name	*Forward Primer Sequence*	*Reverse Primer Sequence*	*Amplicon*
GAPDH	TGA TGA CAT CAA GAA GGT GGT GAA G	TCC TTG GAG GCC ATG TGG GCC AT	240bp
FoxO3a	GCA AAC CAC ACA AAA CCC CG	TGT GCT GTC TTC GGC TCT TC	286bp

3.9 ELISA

3.9.1 Reagents

3.9.1.1 **ELISA Kits**

MMP3	R&D Systems – Human Total MMP-3 Duo Set, #DY513
CXCl9	R&D Systems – Human CXCl9/MIG Duo Set, #DY392
CXCL11	R&D Systems – Human CXCl11/I-TAC Duo Set, #DY672
IL6	eBioscience – Human IL6 ELISA Kit, #88-7066
IL8	eBioscience – Human IL8 ELISA Kit, #88-8086

3.9.2 Devices

3.9.2.1 **ELISA Reader**

Biotek – ELISA Reader Epoch

3.9.3 Harvesting of ELISA Samples

After stimulation, 1.5ml of supernatant was carefully transferred into an eppi and centrifuged for 15’ at 15krpm at 4°C. After centrifugation 1.4ml of the supernatant were gently transferred into a new eppi and stored at -20°C.

3.9.4 ELISA Assays

The ELISA assays were performed according to the manufacturer’s protocol. Technical duplicates and biological triplicates were used for analysis.

3.10 Immunofluorescence

3.10.1 Reagents

3.10.1.1 **Fixation solution**

Paraformaldehyde (PFA)	4%
Tween 20	0.02%
PBS	

3.10.1.2 **Blocking solution**

BSA	5%
Triton X-100	0.3%
PBS	

3.10.1.3 **Triton X-100**

Thermo Fisher – Triton X-100 Surfact-Amps Detergent Solution, #28314

3.10.1.4 **Antibodies**

FoxO3a	Cell signalling – FoxO3a (D19A7) Rabbit mAb, #12829
Goat-anti-rabbit	Invitrogen – Rabbit anti-Goat IgG Superclonal Secondary Antibody, Alexa Fluor 555, # A27017
DAPI	Sigma-Aldrich – DAPI dihydrochloride, # D9542

3.10.1.5 **Fluorescent Mounting Medium**

DAKO – Fluorescent Mounting Medium, #S3023

3.10.2 Devices & Software

3.10.2.1 **Microscope (for cover slides)**

Leica – SP5, MaiTai – titanium Sapphire laser; Leica- LAS-AF Software

3.10.2.2 **Microscope (for automated high content analysis)**

Perkin Elmer – Opera Phoenix 1400L15076

(Software: Harmony (v.4.1))

3.10.2.3 **Imaris Software**

Bitplane – Imaris (v.9.1)

3.10.2.4 **Cellprofiler**

Cell profiler software (v.2.2)[28]

3.10.2.5 **R (inclusive Packages)**

R Software (v.3.4.1)[27]

3.10.3 Preparation: Immunofluorescence on cover glasses

Autoclaved cover glasses were carefully placed in PBS filled wells of a 24-Wells plate. PBS was aspirated and the cells were seeded overnight. After the experiment, the media was removed, the cells were rinsed gently 1x with PBS and fixed with fixation solution for 15 minutes. The fixation solution was removed, rinsed 3x with PBS and blocked with blocking solution at room temperature for 1h. The primary antibody was diluted 1:1000 in blocking solution and 500µl of this primary antibody solution was added to the cells over night at 4°C. After removing of the primary antibody, the cells were rinsed 3x with PBS and the incubated with the secondary antibody for 1h, at room temperature, in the dark. The secondary antibody was removed. The cells were rinsed 3x with PBS and incubated with DAPI 1:5000 in PBS for 2 minutes. After removing the DAPI the samples were stained with phalloidin 1:2500 for 5 minutes and rinsed again 3x with PBS. The cover glass were carefully transferred onto glass slides and sealed with mounting media overnight, at room temperature, in the dark. The cells were stored after sealing on 4°C in the dark.

3.10.4 Analysis: Immunofluorescence on cover glass.

FLS were observed via immunofluorescence microscopy to identify a trend in the nuclear/ perinuclear distribution of FoxO3a under TNFα stimulation. Cell were analysed with a 20x, 40x magnification. Images were taken at 20x magnification in 2 channels (FoxO3a, DAPI) beginning with the weaker signal (FoxO3a) to avoid photo bleaching. Images have been analysed in concern to the cytoplasmic shuttling of FoxO3a under TNFα stimulation.

3.10.5 Preparation: Automated high throughput, high quality confocal laser immunofluorescence microscopy for single cell analysis

96-well plates were pre-treated with 50%FCS in DMEM complete for 24h. 7000 FLS were seeded per well in a 96-well plate. After stimulation, the media was carefully removed by pipetting, the FLS were rinsed 1x with PBS and fixed with fixation solution for 15 minutes. The fixation solution was removed and cells were blocked with blocking solution for 1h. The primary antibody was diluted 1:1000 in blocking solution and 90µl per well of this primary antibody solution was incubated on the cells over night at 4°C. After removing the primary antibody, the FLS were incubated with the secondary antibody for 1h at room temperature in the dark. After incubation the secondary antibody was removed and cells were incubated with DAPI 1:5000 in PBS for 2 minutes. After removing of the DAPI, the cells were washed 3x with PBS and stored in 100µl PBS to avoid shrinking, sealed (to avoid drying), on 4°C in the dark.

3.10.6 Analysis: Automated high throughput, high quality confocal laser immunofluorescence microscopy for single cell analysis

The 96-well plates were analysed in an automated inverse IF microscope. The plates were analysed in 5x5 frames of the centre at a 20x magnification. The images were analysed with the Cellprofiler Software in usage of the afterwards described pipeline. Images of both channels were corrected via an illumination correction (Gaussian Filter, smoothing filter size = 200, calculated for each image). After illumination correction, nuclei areas defined by the DAPI channel Images with a diameter between 17µm and 57µm and perinuclear areas an 11.4µm (8px) wide ring surrounding the nuclei area. Only cells with a neighbourhood distance of 14µm (10px) between the nuclei were selected to avoid overlapping of cells. Intensities of both areas were measured in the FoxO3a channel. For the statistical analysis and data management, the programming language R was used. The mean Intensity value of nuclear and perinuclear localized FoxO3a intensities were used to create a ratio of nuclear / perinuclear intensity of each cell, representing the translocation of FoxO3a. The ratio was log2 transformed to assure a normal distribution. Cells were clustered well-wise and treated as independent sample. Delta of nuclear/perinuclear FoxO3a-intensity ratio was used to verify the alteration of nuclear/perinuclear translocation. Unpaired T-test was used for statistical analysis of the groups.

4 Discussion

RA is a chronic, inflammatory disease with substantial unmet medical needs. Uncontrolled disease results in persistent synovial inflammation, which causes loss-of-function and disability due to cartilage and bone damage [1, 32]. Despite, the initial cause for the synovial inflammation remains unknown yet, the crosstalk between the resident FLS and the infiltrating immune cells are known to dictate the progression of the inflammatory driven joint destruction [1,4,56]. In response to cytokines produced by immune cells (e.g. TNFα), FLS secrete vast amounts of proinflammatory cytokines (e.g. IL6), chemokines (e.g. CXCL9, CXCL10) and tissue degrading enzymes (e.g. MMP3) [60] which actively progress the inflammatory destruction of the joint. These FLS-derived pro-inflammatory mediators perpetuate recruitment, activation and retention of immune cells in the synovial microenvironment [9]. MMPs contribute to irreversible cartilage and bone damage. Therefore, FLS have emerged as potential therapeutic targets for the treatment of inflammatory arthritis. Ongoing efforts are being made to understand how FLS respond to and shape the inflammatory microenvironment [49]. Most studies have concentrated on prototypical TNFα-driven circuits, such as the NF-κB and MAPK pathways. But more recent studies have emphasized a role for other pathways, such as the phosphatidylinositol-3-kinase/Protein kinase B (PI3K/AKT) signalling circuit. This pathway, which is tightly controlled, is crucial to many important cellular processes, such as metabolism, cell growth, proliferation, survival and apoptosis [54]. In response to extracellular signals, PI3K promotes the conversion of phosphatidylinositol (3, 4)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3), which induces the activation of AKT/PKB [54]. Once activated (phosphorylated), AKT shuttles to the nucleus, where it regulates the activity of a wide range of proteins by phosphorylation, including Forkhead box O (FoxO) transcription factors [20]. Persistent activation of the PI3K/AKT pathway was observed in both, animal arthritis models and RA synovial tissues [61, 62]. Perturbations in the PI3K/AKT pathway might contribute to persistent arthritis through inhibition of FoxOs.

Since Genome-wide association studies data could link FoxO3a activity with RA disease severity, FoxO3a maintained more and more attention in the perspective of a potential therapeutic target. A single-nucleotide polymorphism (SNP) in FoxO3a (rs12212067; T>G), which reduced pro-inflammatory (e.g. TNFα, IL-1β), but increased anti-inflammatory (IL-10) cytokine production of monocytes in vitro, was associated with lower RA activity scores and less radiographic progression of RA[35, 36]. We here demonstrate that FoxO3a regulates the inflammatory response of mesenchymal stromal cell (FLS). More specifically our studies reveal that TNFα induced phosphorylation and nuclear exclusion of FoxO3a in RA-FLS. Interestingly, the previously in other cell types described cytoplasmic degradation [45] of inactivated/phosphorylated FoxO could not be observed in RA-FLS. This phenomenon of stable cytoplasmic FoxO3a levels can be explained by potential additional cytoplasmic functions of FoxO3a in RA-FLS and should be subject to further studies. Gene expression profiling revealed a role for FoxO3a in FLS-directed immune cell recruitment and activation. Knockdown of FoxO3a transcriptional function by FoxO3a targeting siRNA pools was mirrored by increased TNFα-induced expression of the pro-inflammatory chemokines CXCL9, CXCL10 and CXCL11. These CXC-motif chemokines are present at elevated levels in synovial tissues and fluids of RA patients [60]. Previous studies assumed that activated FLS might be the primary source of

these chemokines in RA [49]. Regarding their function, CXCL9, CXCL10 and CXCL11, are crucial for leucocyte trafficking during inflammation, especially acting on activated T-cells.

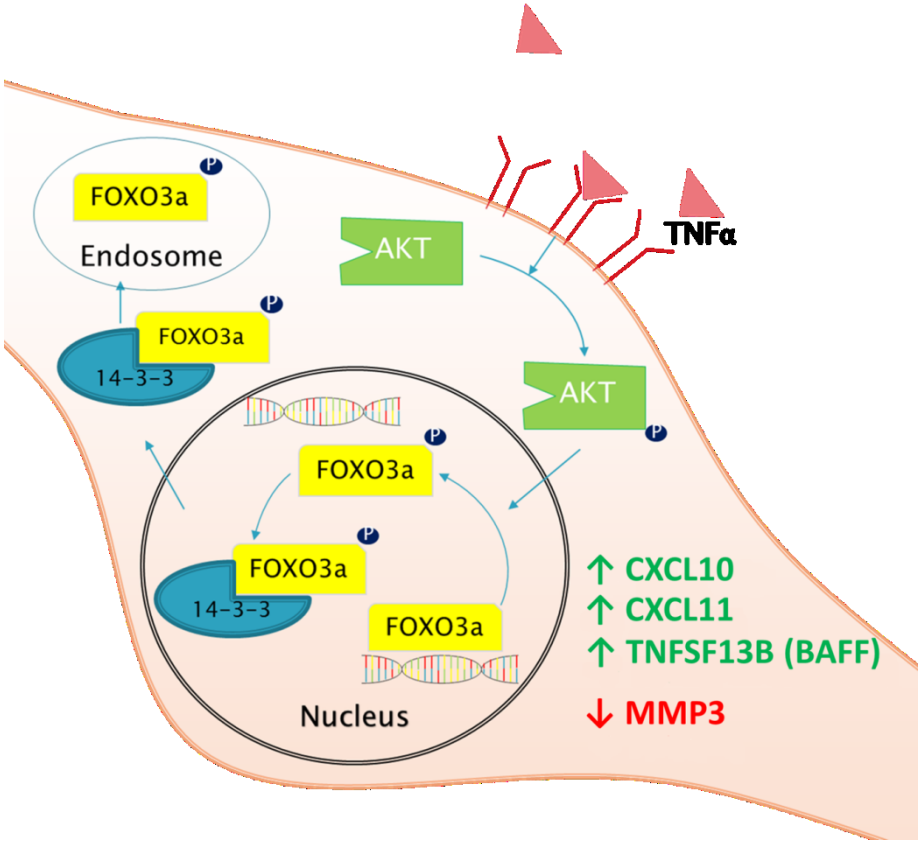

Figure 16: Schematic overview of FoxO3a regulation

Taken together, these data suggest that the here described TNFα/PI3K/AKT/FoxO3a signalling circuit plays an essential role in T-cell recruitment and activation during synovial inflammation. Our findings identify a TNFα-activated signalling circuit that contributes to the pro-inflammatory functions of TNFα and suggest that the PI3K/AKT/FoxO3a axis is important for the ongoing recruitment and activation of leucocytes in the rheumatoid synovium.

Interestingly, we found decreased expression of the detrimental MMP3 in FoxO3a knockdowned RA- FLS. Thus, FoxO3a inactivation during the course of inflammation might have beneficial (e.g. inhibition of CXCL10 expression) but also detrimental effects by increasing the secretion MMPs. This has to be considered in clinical trials investigating inhibition of the PI3K/AKT/FoxO pathway. Our studies reveal a previously unknown TNFα-activated pathway that contributes to the TNFα-induced inflammatory response in FLS. Therefore, our work on FoxO3a provides novel insights into the molecular mechanism underlying TNFα-driven inflammation.

5 Table of figures

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