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Arbeitsbereich Orthopädie der Medizinischen Fakultät Charité –

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DISSERTATION

Cytokine-mediated expression of catabolic & pro-inflammatory mediators in synovial fibroblasts with regard to the pathogenesis of osteoarthritis

Zur Erlangung des akademischen Grades

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List of abbreviations

ABI – Applied Biosystems

Ad5 – adenovirus type 5

APS – ammonium peroxodisulphate

BAC – β-actin

BP – base pair

BSA – bovine serum albumin

CAR – coxsackie virus B adenovirus receptor

CD44 – cluster of differentiation 44 (=hyaluronan-receptor)

cDNA – complementary DNA

CMV - cytomegalovirus

Ct – cycle of threshold

DAF – decay-accelerating factor (=CD55)

DAPI – 4', 6-diamidino-2-phenylindol

DMEM – Dulbecco's modification of Eagle's medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

dsDNA – double-stranded DNA

DTT – 1,4-dithio-DL-threitol

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EGTA – ethylene glycol tetra acetic acid

EIA – enzyme immunoassay (=ELISA)

ELISA – enzyme-linked immunosorbent assay

ER – endoplasmic reticulum

ERM – ezrin, radixin and moesin

FACS – fluorescence activated cell sorting

FC – flow cytometry

FCS – fetal calf serum

FITC – fluorescein isothiocyanate

FRET – fluorescence resonance energy transfer

GFP – green fluorescent protein

HA – hyaluronan (hyaluronic acid)

HBSS – Hank's balanced salt solution

HEPES – 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid

HRP – horse radish peroxidase

ICAM-1 – inter cellular adhesion molecule 1 (=CD54)

IF – immunofluorescence

IFN – interferon

Ig – immunoglobulin

IGF-1 – insulin-like growth factor-1

IgG – immunoglobulin G

IL – interleukin

IL1-R – interleukin 1 receptor

ILK – integrin linked kinase

MAB – monoclonal antibody

MMP – matrix metalloproteinase

mRNA – messenger RNA

MSC – mesenchymal stem cell

NO – nitric oxide

OA – osteoarthritis

PBS – phosphate buffered saline

PDGF-R – platelet derived growth factor receptor

PE – phycoerythrin

Pen/Strep – penicillin/streptomycin

PFA – paraformaldehyde

PG - prostaglandin

PVDF – polyvinylidene fluoride

RA – rheumatoid arthritis

rER – rough endoplasmic reticulum

RNA – ribonucleic acid

RTD-PCR – real time detection-polymerase chain reaction

SDS – sodium dodecyl sulfate

SF – synovial fibroblast(s)

SM – synovial membrane

SP-A – surfactant protein A

SV40 Tag – simian vacuolating virus 40 T-antigen

TAE – tris base, acetic acid & EDTA

TBP – TATA box binding protein

TBS – TRIS-buffered salt solution

TEMED – tetramethylethylenediamine

TEP – total endoprosthesis

TGF-β – transforming growth factor-β

TH – T-helper

TIMP – tissue inhibitor of matrix metalloproteinases

TNF-R1 – tumor necrosis factor-α receptor 1

TNFα – tumor necrosis factor α

UDPGD – uridine diphosphoglucose dehydrogenase

UNG – uracil-DNA glycosylase

VCAM-1 – vascular cell adhesion molecule-1

VEGF – vascular endothelial growth factor

WB – western blot

Kurzzusammenfassung

Hintergrund: Die Synovialmembran (SM) ist Bestandteil jeder Diarthrose und potentieller Knotenpunkt in der Arthrose-Pathogenese. Pro-inflammatorische Zytokine, insbesondere Tumornekrosefaktor α (TNFα), die in arthrotischen Gelenken in erhöhter Konzentration nachweisbar sind, könnten die Synthese zusätzlicher pro-inflammatorischer und kataboler Mediatoren, z.B. Interleukin 6 (IL-6), in Synovialfibroblasten (SF) induzieren und zu einem Teufelskreis führen, der die Knorpelschädigung und Chondrozytenapoptose beschleunigt. Die Wirkungen intra-artikulärer Zytokine auf SF wurden noch nicht vollständig verstanden. Daher war es Ziel dieser Dissertation das Wechselspiel zwischen TNFα und IL-10 in SF zu charakterisieren und zu entscheiden, ob IL-10 dessen katabole Effekte modulieren bzw. blockieren kann.

Methoden: Aus Kniegelenks-SM betroffener Spender wurden primäre menschliche SF isoliert, kultiviert, expandiert und mit TNFα, IL-10 oder TNFα + IL-10 für 24 h stimuliert. Die Genexpressionen von IL-6, IL-10 und Matrixmetalloprotease 1 bzw. 3 (MMP-1; MMP-3) wurden mittels RTD-PCR bestimmt. Die Proteinsynthese derselben Mediatoren wurde per Durchflusszytometrie (FC) und Immunfluoreszenzfärbung (IF) untersucht, die Proteinsynthese von Kollagen I, CD44 und β_1 Integrin mit Western Blot (WB) nachgewiesen. Ferner wurden SF mit adenoviralen IL-10 Überexpressionsvektoren transduziert um einen potenziellen Therapieansatz bei Arthrose zu simulieren. Die Auswirkungen der Transduktion wurden per RTD-PCR überprüft und zusätzlich die IL-10 Proteinsekretion mittels Enzyme-linked immunosorbent assay (ELISA) untersucht. Abschließend wurde die permanente Zelllinie K4IM etabliert um für zukünftige Experimente die komplizierte Zellisolierung, langsame Expansion und inter-individuelle Unterschiede zu eliminieren.

Ergebnisse: Kultivierte SF wurden durch TNFα bzw. TNFα + IL-10 aktiviert und steigerten die Genexpression von IL-6, IL-10, MMP-1 & -3. Die Untersuchung der Proteinsynthese via IF zeigte Resultate, die mit denen der Genexpression übereinstimmten. Die Ergebnisse der FC zeigten Abweichungen zu den RTD-PCR Beobachtungen.

Die durchgeführten WB wiesen nach, dass die Zytokinstimulation auch Auswirkungen auf extrazelluläre Matrixproteine und -rezeptoren hatte, die im Einzelnen sehr unterschiedlich ausfielen.

Die adenovirale Transduktion mit IL-10 war erfolgreich. Dessen Genexpression wurde stark gesteigert, aber die Effekte auf die Expression von IL-6, MMP-1 undMMP-3 waren statistisch nicht signifikant.

Die K4IM Zelllinie zeigte meist Reaktionen, die mit denjenigen kultivierter humaner SF übereinstimmten.

Schlussfolgerung: Kultivierte humane SF werden durch TNFα und TNFα + IL-10 stark aktiviert und steigern die Expression pro-inflammatorischer und kataboler Mediatoren. Diese Ergebnisse werfen mehr Licht auf die Rolle, die SF in der Arthrose-Pathogenese spielen und lassen diese Zellen als Ziel für zukünftige Therapien erscheinen, auch wenn eine IL-10 Überexpression unzureichend für eine effektive Therapie der Osteoarthrose sein dürfte. Die K4IM Zelllinie könnte in zukünftigen Stimulationsexperimenten als passendes Modell für kultivierte humane SF dienen.

Abstract

Background: The synovial membrane (SM) is part of every diarthrosis and potential hub in the pathogenesis of osteoarthritis (OA). Pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNFα), which are detectable in osteoarthritic joints, might induce the synthesis of pro-inflammatory and catabolic mediators, e.g. interleukin 6 (IL-6), in synovial fibroblasts (SF), leading to a vicious circle that could accelerate cartilage degradation and chondrocyte apoptosis. The effects of intra-articular cytokines on SF are not completely understood. Hence, it was the aim of this dissertation to characterize the interplay between TNFα and the anti-inflammatory IL-10 in SF to decide whether IL-10 might modulate or block its catabolic effects.

Methods: Primary human SF were isolated from knee joints of OA-affected donors, cultured, expanded and stimulated with TNFα, IL-10 or TNFα + IL-10 for 24 h. Gene expression of IL-6, IL-10, matrix metalloproteinases-1 & -3 (MMP-1; MMP-3) was investigated via RTD-PCR. Protein synthesis of the same mediators as well as type I collagen, CD44 and β_1 integrin was determined using flow cytometry (FC), immunofluorescence labeling (IF) and western blot (WB). IL-10 adenoviral transduction was performed to simulate a possible OA treatment. The transduction effects were measured using RTD-PCR and investigation of IL-10 protein secretion via an enzyme-linked immunosorbent assay (ELISA). Finally, the permanent K4IM cell line was established as a possible substitute for cultured human SF in future experiments.

Results: Cultured SF were activated by TNFα and TNFα + IL-10, increasing their gene expression of IL-6, IL-10, MMP-1 & -3. Investigation of the protein synthesis via IF showed results consistent with RTD-PCR, while FC revealed distinct differences. WB demonstrated varied stimulation effects on the synthesis of extracellular matrix proteins and matrix receptors. The adenoviral transduction was successful. The gene expression of IL-10 was greatly elevated. Effects on the gene expression of IL-6, MMP-1 and MMP-3 were statistically not significant. Reactions in the K4IM cell line were largely congruent with those observed in cultured human SF.

Conclusion: Human SF in culture are strongly activated by TNFα and TNFα + IL-10, increasing the gene expression and protein synthesis of pro-inflammatory and catabolic mediators. These findings illuminate the role of SF in the pathogenesis of OA, suggesting them as a potential future therapeutic target, even though IL-10 overexpression alone appears to be insufficient for an effective OA therapy. The K4IM cell line could be a suitable substitute for cultured human SF in future stimulation experiments.

1. Introduction

1.1 The synovial membrane and its composition

The synovial membrane (SM) is an integral component of all synovial joints in the human body, covering every articular surface not occupied by cartilage, disci or menisci. It consists of a cellular layer directly adjacent to the joint cavity (*Lamina synovialis intima*) and a layer of loose, highly vascularized connective tissue (*Lamina synovialis subintima*), which connects the synovial membrane to the fibrous capsule of the joint (*Lamina fibrosa*) (01) [Fig. 1.1].

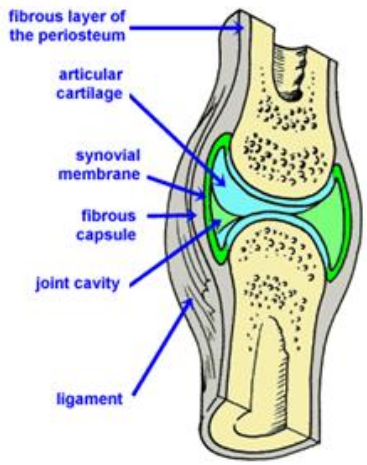


Fig. 1.1: Principal components and structure of a synovial joint
The articulating surfaces are covered with cartilage, which serves as a shock absorber and facilitates smooth joint movement. The joint cavity is encompassed by a capsule whose internal layer contains the synovial membrane and whose external layer is composed of fibrous tissue (modified after zoology.ubc.ca).

The *Lamina synovialis intima* contains macrophage-like type A synoviocytes, whose precursors are bone marrow derived and whose primary function is phagocytosis, as well as fibroblast-like type B synoviocytes which synthesize and secrete collagen, fibronectin, glycoproteins, lubricin, hyaluronan (HA), proteoglycans, chondroitin-6-sulphate, diverse cytokines and growth factors (02).

Type B synoviocytes are partly located beneath the type A synoviocytes, but are able to reach the joint cavity with their long cytoplasmic extensions to exercise their mainly secretory functions (03). They are primarily responsible for the formation of extracellular matrix (ECM) within the *Lamina synovialis intima* and for the secretion of synovia, a viscous mixture of HA, glycoproteins, glucose, water and desquamated cells of the *Lamina synovialis intima*, which plays an important role in cartilage nutrition, lubrication, maintenance of normal joint functions and intercellular communication (01). These cells are also known as synovial fibroblasts (SF).

1.2 Characteristics of synovial fibroblasts in cell culture

Cultured SF generally appear as elongated, sometimes oval or polygonal cells with twig- or branch-like cytoplasmic extensions. Occasionally, dendritic or stellar cells can also be found [Fig. 1.2] (04).

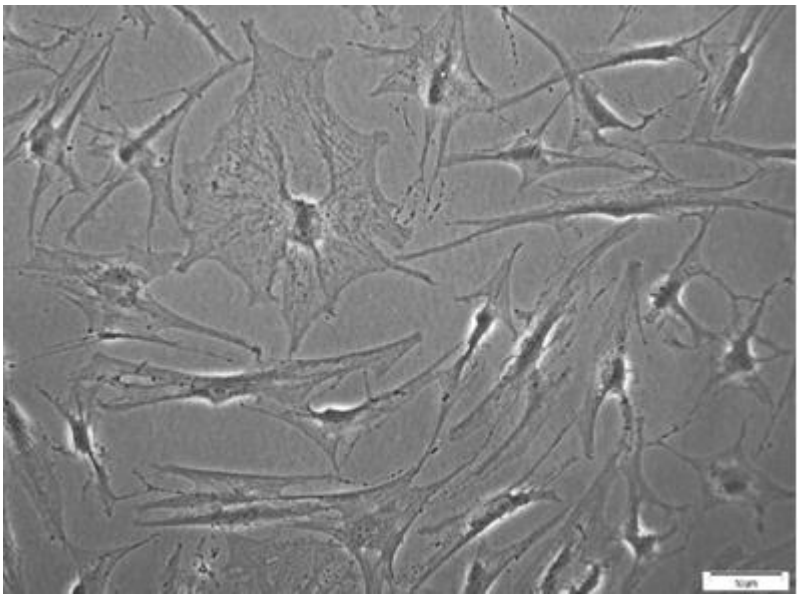


Fig. 1.2: Bright-field microscope photography of cultured human SF

These SF in cell culture (passage 5) show the typical elongated morphology including twig-like cytoplasmic extensions. Note the "bat-like" cell in the left upper quadrant of the picture. This morphology of human SF in culture can sometimes be observed, mostly in later passages.
Scale bar: 50 µm. The picture is a courtesy of Ms. Nadine Jork, AG Schulze-Tanzil.

The cellular ultrastructure of SF reveals a high content of rough endoplasmic reticulum (rER) as well as an abundance of Golgi complexes, „lamellar bodies" and secretory granules due to the secretory functions of these cells (01;04). Lamellar bodies can also be found in type II pneumocytes. They contain surfactant phospholipids and surfactant protein A (SP-A) which decrease the surface tension within the alveoli. In the joint, the surfactant serves as a lubricant to facilitate smooth, gliding movements of the articulating surfaces (01).
This fact underlines the enormous importance of SF and their secretory products for normal joint function.
SF do not express unique surface markers, but the combination of vascular cell adhesion molecule-1 (VCAM-1), decay accelerating factor (DAF), uridine diphosphoglucose dehydrogenase (UDPGD) (02) and the hyaluronan receptor (CD44) as well as their typical appearance can be used to identify them (01).

1.3 The K4IM synovial fibroblast cell line

K4IM is a permanent cell line with unlimited proliferative potential, established from cultured human SF (05). These cells were extracted from the knee joint synovium of a healthy donor and immortalized via transduction with simian vacuolating virus 40 T-antigen (SV40 Tag) (05). Post-transduction, the immortalized cells maintained intercellular adhesion molecule 1

(ICAM-1), CD44 and Fas (CD95) expression but lost VCAM-1, interleukin 1 receptor (IL-1-R) and platelet derived growth factor receptor (PDGF-R) expression (05).

K4IM cells show the typical morphology of SF in cell culture. However, they are smaller in size when compared to primary human SF and cultured human SF and do not grow in an ordered confluent monolayer but rather in multilayers if given the chance (05) [Fig. 1.3].

Their mitotic activity is approximately 5 times higher than that of human SF in cell culture and the gene expression in response to TNFα or 20% fetal calf serum (FCS) was not showing any significant difference in comparison to the wild type SF gene expression when the cell line was established (05).

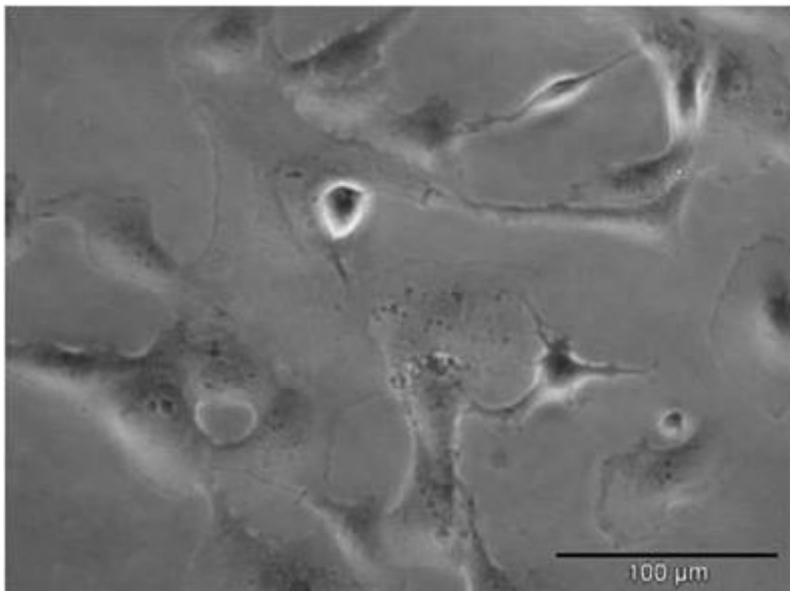


Fig. 1.3: Bright-field microscopic photography of K4IM cells in cell culture

The K4IM cells show similar features to primary human SF and cultured human SF, but are often considerably smaller in size and tend to overgrow each other. Notice the cell lump in the left lower quadrant, where it becomes impossible to distinguish between single cells due to overgrowth. Scale bar: 100µm.

This photograph is a courtesy of Ms. Katharina Gorte, AG Schulze-Tanzil.

The rapid growth rate as well as the fact that K4IM cells are derived from cultured SF of a healthy donor could make them a valuable resource for further investigation of the early stages of RA and OA (05). Especially since regularly used SF are usually extracted during operations in the late stages of these diseases and may have undergone physiologic changes due to the constant exposure to a pro-inflammatory environment and/or continuous treatment (05). Additionally, the relative lack of donors, the finite live span of human SF in cell culture as well as their relatively slow mitotic rate and inter-individual gene expression differences may be by-passed by using the permanent K4IM cell line in the future, since these cells can be bred fast, in infinite numbers and reproducibly analyzed (05).

1.4 Osteoarthritis

Osteoarthritis (OA) is a degenerative and inflammatory joint disease leading to progressive and irreversible destruction of the articular cartilage (06). There is a well-established distinction between two subtypes, primary (i.e. idiopathic) and secondary OA. The former develops, as the name suggests, without any definitive cause while the latter occurs due to specific reasons such as joint deformities, joint misalignment, misuse, overuse, overload, chronic arthritis, metabolic changes in the joint tissues, chronic gout or chronic-recurrent intra-articular hemorrhages (06). Several contributing or facilitating risk factors for the development of OA have been identified. These are primarily: ageing, female gender, joint trauma, genetic causes and obesity (07). As suggested by the risk factors, the prevalence increases steadily with age and women are more often affected than men (08). The most commonly affected joints are hip and knee joints, which has a crippling effect on the subject's mobility (06).

Regardless of etiology, the early stages of OA are always characterized by disrupted joint homeostasis. Classically, the disease commences in the cartilage but it has been proven that OA can just as well start in the bone or the SM (07). As the disease progresses, the above mentioned components interact on the cytokine level, which induces synovitis, cartilage destruction and formation of osteophytes, thereby slowly leading to total joint destruction (07).

1.5 The synovial membrane's potential role in the pathogenesis of osteoarthritis

In classic OA, the damaged cartilage secretes most of the pro-inflammatory cytokines (e.g. interleukin-1β [IL-1β], IL-6 or tumor necrosis factor α [TNFα]), radicals (e.g. nitric oxide [NO]) and matrix metalloproteinases [MMPs] (07). This cocktail of local messengers and enzymes leads to chondrocyte apoptosis and degradation of ECM within the articular cartilage (07). It also causes hypertrophy, hyperplasia and inflammation of the SM, called synovitis, especially in areas where the SM borders on damaged cartilage (07). As part of this process, intimal cells proliferate and sub-intimal cells (e.g. SF) infiltrate the *Lamina synovialis intima*, while lymphocytes accumulate within the SM. These white blood cells produce additional pro-inflammatory cytokines (07). The infiltrating SF have been shown to develop aggressive potential. They actively invade the articular cartilage, destroy ECM and are supposed to secrete angiogenetic factors, especially VEGF (09). Due to these angiogenetic factors, the number of capillaries and total circulation within the SM increase and more lymphocytes can reach the inflamed joint, which further increases the cytokine levels (07, 10). Additionally, it has been postulated that SF within the inflamed parts of the SM may produce pro-inflammatory cytokines and MMPs themselves, which in turn would hasten cartilage destruction even more (07).

In the final stages of OA, the subchondral bone is affected, too (07). It starts to degenerate and bone cells contribute further cytokines and radicals to the ongoing disease process. Additionally, reactive anabolic growth factors (e.g. IGF-1 and TGF-β) cause osteophyte formation and remodeling of the remaining subchondral bone which finalizes the complete destruction of the joint (07).

All in all, the picture of a possible vicious circle reveals itself. This would classically involve chondrocytes and cells within the bone on the one hand and cells of the synovium on the other hand (07). The postulated interrelation of intra-articular cytokines secreted by chondrocytes, their effects on SF and *vice versa*, especially on the cytokine release, has not yet been completely understood (07). However, this reciprocal interaction may present a possible weak spot in the vicious circle and thereby a way to devise a treatment for preventing joint destruction in future OA patients [Fig. 1.4].

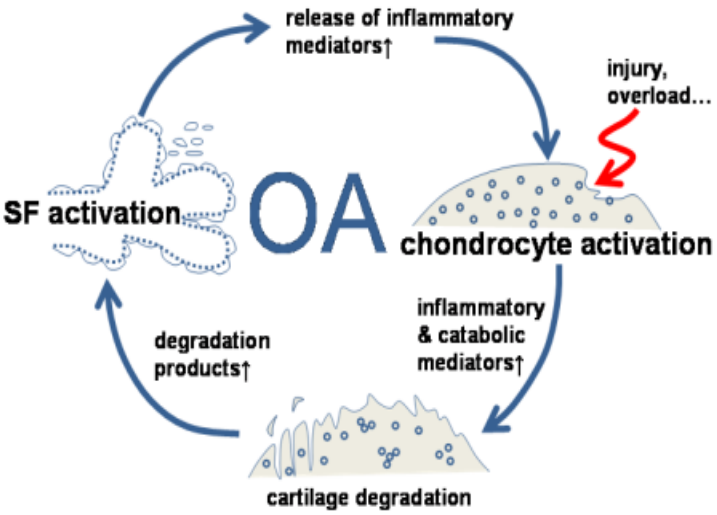


Fig. 1.4: The suspected vicious circle of "classical" osteoarthritis (OA)
Initial injury of the articular cartilage causes chondrocyte activation and the liberation of pro-inflammatory & catabolic mediators. Degradation products released from the cartilage activate SF within the synovial membrane, which leads to further production of pro-inflammatory mediators. Additional chondrocyte activation and continuous destruction of the cartilage ensue. More SF are activated until the joint cartilage is completely destroyed.

1.6 Cytokines and matrix metalloproteinases in this study

Cytokines are essentially a collection of glycoproteins with low molecular weight (11). These glycoproteins act as messengers between cells and can be divided into different subpopulations: interleukins (ILs), interferons (IFNs), chemokines and growth factors. ILs, IFNs and chemokines are essential for a normal immune system function, while growth factors have enormous importance in- as well as outside of the immune system (11).

MMPs are enzymes capable of degrading components of the ECM. These enzymes are involved in entirely catabolic processes as well as in remodeling of the ECM throughout the entire body and are implicated to be an important factor in the pathogenesis of inflammatory arthritides of many different etiologies (12).

1.6.1. TNFα

TNFα is a pro-inflammatory cytokine possessing catabolic and cytolytic effects against tumor cells and is an important mediator in the defense against bacterial, viral and parasitic infections (13). It is primarily produced by monocytes, macrophages and T-cells and stimulates proliferation as well as activation of macrophages (13). The mechanism, by which the two opposing pathways are regulated, leading to catabolism on the one hand and anabolism on the other, is not yet understood (13, 14). However, previous studies on primary human chondrocytes conducted in our research group seem to indicate that TNFα activates the TNFα receptor 1 (TNF-R1), which contains an 80 amino acid long death domain, thereby stimulating intrinsic apoptotic pathways in order to mediate some of its catabolic effects (14). The result is an increase in caspase activity as well as bax/bcl-2 ratio and a decrease in collagen synthesis, ultimately resulting in cell death (14). Though this idea is entirely speculative, it seems probable that TNFα's effects on tumor cells are similar.

Because of these pro-apoptotic and catabolic effects on chondrocytes, TNFα also seems to be important for the pathogenesis of OA, especially since it increases cartilage catabolism, suppresses the synthesis of cartilage ECM, promotes synovitis and stimulates the expression of matrix metalloproteinases (MMPs) (15).

1.6.2. IL-6

IL-6 is an ambivalent cytokine having both pro-inflammatory and anti-inflammatory effects (16). It is an important mediator in the body's immune response, especially against bacterial pneumonia pathogens, e.g. *Streptococcus pneumoniae* and *Listeria monocytogenes* (17). This cytokine is also thought to increase the production of acute phase proteins and to serve as an endogenous pyrogen (17). Expression of IL-6 in chondrocytes and SF is induced by TNFα (18). According to current research, IL-6 is thought to inhibit excessive production of TNFα and possibly of IL-1, hence it supposedly has anti-inflammatory properties as mentioned above (17).

Despite its possibly ambivalent role in the immune response, this cytokine has classically been proposed as a contributor to the pathogenesis of OA by increasing the number of inflammatory cells in synovial tissue and amplifying the effects of other pro-inflammatory cytokines, leading to increased synthesis of MMPs and inhibition of proteoglycan production (18). Furthermore, it is reported to cause a down regulation of type II collagen gene expression in chondrocytes, which leads to a decrease in the synthesis of ECM (18).

However, there is also some evidence that IL-6 induces the production of tissue inhibitor of metalloproteinases (TIMP), thereby limiting the proteolytic damage caused by MMPs (18).

Additionally, animal trials have shown that IL-6 deficient mice developed age-related OA more often and that the cartilage repair response in these mice was impaired (19).
All in all, the role of IL-6 in the development of OA remains controversial and requires further investigation.

1.6.3. IL-10

IL-10 is primarily produced in TH_2-cells. It inhibits macrophage-mediated, pro-inflammatory immune reactions, while stimulating and activating B-lymphocytes and plasma cells, thereby supporting a humoral immune response (20). IL-10 is generally regarded as an antagonist to TNFα and seems to counteract its catabolic and pro-apoptotic effects on chondrocytes by increasing the bcl2/bax ratio and blocking TNFα's deleterious effect on the ECM (14). This cytokine is also produced by chondrocytes and its levels are elevated in OA joints, which indicates a possible reaction of the body in order to limit or end the inflammatory processes within the joint (14, 20, 21). The exact interplay between IL-10 and TNFα in connective tissue cells has not yet been understood. However, results of adenoviral IL-10 transduction experiments on primary human chondrocytes obtained in our research group seem to suggest IL-10 overexpression as a possible therapeutic approach in the future treatment of OA (21).

1.6.4 Matrix metalloproteinase-1

MMP-1, also known as collagenase-1, is generally produced by fibroblasts as well as chondrocytes and SF at sites of synovial attachment to articular cartilage (22). It is one of the key enzymes acting in fibrolysis, a process closely related to tissue remodeling (23) and is capable of degrading the interstitial collagen types I, II and III (24). Therefore, MMP-1 is thought to play an important role in tissue morphogenesis, tissue remodeling and wound repair (24).
It is excessively expressed in the earlier stages of OA, especially in superficial chondrocytes due to induction by pro-inflammatory cytokines and degrades the local ECM, thereby leading to cartilage destruction (24). MMP-1 expression decreases in late stage OA, most likely because there are less chondrocytes left to produce the enzyme (24).

1.6.5 Matrix metalloproteinase-3

MMP-3 or stromelysin-1 is an enzyme involved in the breakdown of ECM in physiologic tissue remodeling, angiogenesis and embryologic development as well as in tumor metastasis and inflammatory arthritis (12). Just as most other MMPs, it is secreted as an inactive zymogen and activated when cleaved by extracellular proteinases. MMP-3 is capable of degrading collagen types II, IV and IX, proteoglycans, laminin, fibronectin, gelatins and elastin (25).

Additionally, it can activate other MMPs such as MMP-1 (25). The expression of MMP-3 is primarily regulated on the level of transcription and the enzyme can be induced by growth factors and cytokines, such as TNFα or IL-6 (12, 25). An over-expression of MMP-3 is implicated in the pathogenesis of progressive ECM destruction in inflammatory arthritis of any etiology (25). In RA, its synovial levels are greatly increased and it is positively correlated with disease activity. That makes this enzyme a sensitive marker of cytokine-driven local inflammation and a potential target for therapeutic interventions (12). Because MMP-3 is not a specific marker for RA, the same principles as in RA might apply to the diagnosis and potential management of OA (12), which warrants further investigation of this enzyme's activity in OA joints.

1.7 Type I collagen, β_1 integrin and CD44

1.7.1 Type I collagen

Type I collagen is the most abundant ECM protein in vertebrates and major component of the ECM of skin, bones, ocular sclerae, ligaments, tendons and the *Lamina fibrosa* of the joint capsules (26, 27, 28). It is essential for the provision of mechanical strength in these tissues. However, the abnormal accumulation of type I collagen is also primarily responsible for the major dysfunctions of affected organs in fibrotic processes (28).

The protein is synthesized as type I procollagen by a number of discrete cell types including fibroblasts, osteoblasts and odontoblasts (28). Type I procollagen is coded by two structural genes: COL1A1 and COL1A2 (26). These two genes are located on chromosome 17 and chromosome 7 respectively and encode the pro-α-1 and pro-α-2 chains.

Each type I procollagen molecule contains two pro-α-1 chains and one pro-α-2 chain. Accordingly, these chains are synthesized in a 2:1 ratio (29).

The protein precursor undergoes extensive post-translational modifications within the rER and Golgi apparatus before being transported out of the cell. After deposition in the pericellular environment, the N-terminal and C-terminal pro-peptides are cleaved and the type I tropo-collagen molecules spontaneously aggregate to form fibril structures (28, 29).

Then these fibril structures form fibers. They are resistant to most proteases but can be degraded by MMPs such as MMP-1 (24).

In vitro experiments using type I collagen-producing cells have shown that different soluble molecules can modulate type I collagen synthesis. In particular, cytokines, including IL-1 and IL-4, transforming growth factor-beta (TGF-β) and insulin-like growth factor-1 (IGF-1), appear to increase the collagen synthesis while other factors such as TNFα, IL-10, IFN-γ, prostaglandin

E2 (PG-E2) and corticosteroids are thought to inhibit type I collagen production in fibroblastic cells (28).

1.7.2 β_1 integrin

Integrins are a superfamily of cell adhesion receptors that provide a structural link between proteins of the ECM (e.g. fibronectin, laminin and collagen) and the cell interior (30). They also modulate a variety of intracellular signaling cascades through interactions with cytoskeletal, adaptor and signaling proteins upon binding extracellular ligands (30, 31, 32, 33). These modulations affect cell behavior such as adhesion, proliferation, survival or apoptosis, cell shape, polarity, motility, gene expression and differentiation (32, 33).

Integrins are heterodimeric transmembrane proteins which consist of α- and β-subunits (33). At least eighteen α- and eight β-subunits are known in humans (33). Each subunit consists of a large extracellular portion, a transmembrane region and a short cytoplasmic domain of 20-50 amino acids (30, 32). These short cytoplasmic tails, especially those of β-subunits, interact with cytoskeletal proteins such as the actin cross-linking proteins talin, α-actinin and filamin A, thereby triggering specific responses within the cell (30, 33). Talin, α-actinin and filamin A have also been implicated in the linking of actin stress fibers to the cell membrane at specialized structures, known as focal contacts, which are formed at cell–substrate contact sites and important for anchoring adherent cells (30, 32).

β_1 integrin, also known as CD29, is coded by a gene on chromosome 10 (33). It plays a crucial role in cell survival and tissue homeostasis through the associated integrin linked kinase (ILK) (34). β_1 integrin binds to the ILK via its cytoplasmic domain and the integrin-activated ILK induces anti-apoptotic signals (34). β_1 integrin forms at least 12 different kinds of integrins via binding to different α-chains of the integrin family, thereby achieving affinity to several ECM components other than fibronectin (34). The possible up- or down-regulation of this important transmembrane protein through cytokines may have effects on the survival of SF in OA joints.

1.7.3 CD44

CD44 is a ubiquitously expressed transmembrane glycoprotein and cell surface adhesion molecule involved in cell-cell and cell-ECM interactions (35). Its principal ligand is HA, a glycosaminoglycan and integral component of the ECM, which increases the viscosity of the synovia and is very important for the proper lubrication of articular surfaces (35). Other CD44 ligands include osteopontin, a chemokine implicated in migration and metastasis of tumor cells, collagen, fibronectin and laminin (36). The major physiological role of CD44 is to maintain three-dimensional organ and tissue structure via cell-cell and cell-matrix adhesion but certain

variant isoforms can also mediate lymphocyte activation, homing and the presentation of chemical factors and hormones (36). Additionally, the receptor plays an important role in wound healing, embryonic cell migration and angiogenesis, where it mediates cell movement (36). It can also induce inflammatory gene expression, especially in macrophages (36).

The human CD 44 gene on chromosome 11 contains nineteen exons, with the first five and the last five being constant (37). The middle nine exons may be alternatively spliced to give rise to multiple variant CD44 isoforms, which, along with the standard CD44 isoform with no variable exons, make up the CD44 class of receptors (37). Post-translational modifications further contribute to the variety of CD 44 receptors (37). The proteins are single-chain molecules comprising an N-terminal extracellular domain, a transmembrane portion and a cytoplasmic C-terminal tail (36, 37).

The main role of the cytoplasmic domain is the transduction of signals from extracellular stimuli. It binds to a number of intracellular proteins including ankyrin and the ezrin, radixin and moesin (ERM) family (36). Ankyrin and ERM proteins connect elements of the plasma membrane with the actin filament network of the cell and thus a direct link between CD44 and intracellular scaffold structures can be envisioned, facilitating cell motility and migration, as well as determining membrane localization of CD44 (36).

1.8 Adenoviruses and transduction with adenoviral vectors

The 57 known serotypes of adenoviruses are a family of double stranded DNA (dsDNA) viruses without an envelope (38). They measure approximately 90nm in diameter and produce a progeny virus within the nucleus of the host cells which is released upon cell lysis (39). These viruses were first isolated in 1953 as respiratory pathogens (40). However, they can cause an entire array of usually acute but self-limited respiratory, gastrointestinal, renal, urinary tract and ocular surface infections (40).

Adenoviruses infect cells via the coxsackie virus B adenovirus receptor (CAR), a transmembrane protein and member of the immunoglobulin superfamily (39). This receptor is expressed on a wide variety of cells including cells of the pancreas, peripheral and central nervous system, respiratory, endothelial and epithelial cells and seems to be up-regulated in inflammation (39).

After binding to the CAR, the adenovirus enters the host cell via clathrin-dependent endocytosis and is transported towards the nucleus where the viral replication takes place (39) [Fig.1.5].

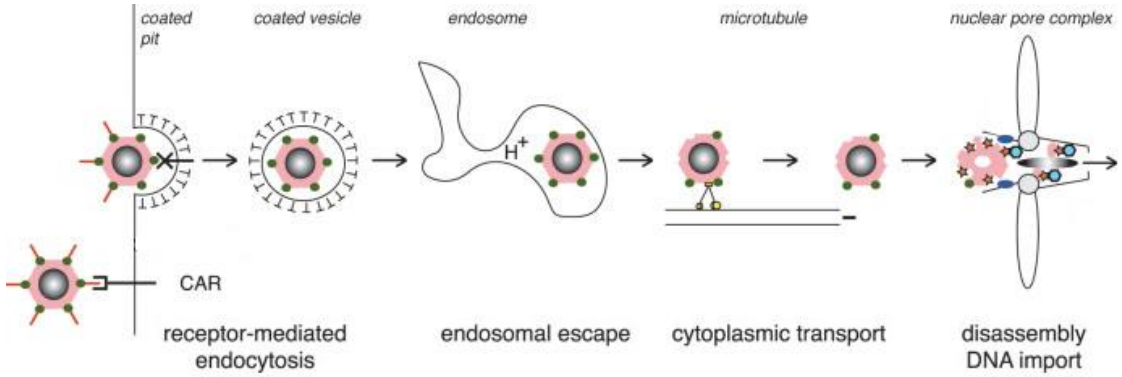


Fig. 1.5: Adenoviral infection of a host cell

The adenovirus binds to CAR and enters the cell via clathrin-dependent, receptor-mediated endocytosis. The coated vesicle fuses with an endosome, a lysosome develops. The coated vesicle is denatured. The virus remains intact, escapes the lysosome (mechanism unknown) and enters dynein-mediated retrograde transport via microtubules. After reaching the nucleus, it is disassembled at the nuclear pores and the DNA is imported into the nucleus, where its transcription takes place and progeny virus is created. (Image modified after Meier O, Greber UF, Adenoviral endocytosis [2004]).

Especially the species C adenoviruses are commonly used as gene transfer vectors in transduction experiments (39). They have been successfully tested in different cell types including primary human mesenchymal stem cells (MSCs) and chondrocytes (38).

The general idea behind adenoviral transduction is to make the target cells express a specific gene and produce the corresponding protein in order to treat a certain, mostly chronic condition like RA, OA or gene deficiencies (41). The possible advantages of therapeutic transductions include bypassing the necessity of systemic exposure with the therapeutic agent and thereby reduction of possible side effects (41). Furthermore, the problem of sufficient delivery to the target tissues is solved (41).

In essence there are two approaches: transduction *in vivo* or *in vitro* with following reintroduction of the transduced cells (41). The direct approach would be more desirable for an easy, inoculation-based clinical application but it may prove difficult to achieve sufficient transduction *in situ* and the transduction would not be target-specific (41). The indirect approach on the other hand allows for controlled transduction as well as selection of cells that express the desired target gene but would take more time and introduce some technical challenges regarding the transplantation or re-implantation of transduced cells, e.g. SF (41).

1.9 Aim of this study

Better understanding of the pathogenesis of OA might reveal possible starting points for future (anti-)cytokine therapies to control the inflammation and stop or delay cartilage destruction within OA joints. Therefore, it was the primary aim of this study to analyze the response of SF to cytokine stimulation with TNFα, IL-10 or the combination of TNFα + IL-10.

For this purpose, the SF were isolated from OA synovial membranes, cultured in monolayer cultures and then stimulated with recombinant TNFα, IL-10 or TNFα + IL-10 for 24 hours. In order to characterize the interplay between the two cytokines, the gene expression and protein synthesis of MMP-1, MMP-3, IL-6 and IL-10 after stimulation were measured using RTD-PCR, flow cytometry (FC) and immunofluorescence (IF) labeling. The PCR primers for those MMPs and cytokines as well as a working protocol for intracellular antibody-mediated staining of SF had to be established first.

The stimulation effects on the protein synthesis of β_1 integrin, CD44 and type I collagen were determined using Western blot (WB).

Additionally, the adenoviral transduction of SF as model for a possible future treatment of OA was tested using an adenoviral overexpression vector for the cytokine candidate IL-10. Post-transduction gene expression analysis of MMP-1, MMP-3, IL-10 and IL-6 via RTD-PCR was performed and the post-transduction IL-10 protein synthesis was assessed using an enzyme-linked immunosorbent assay (ELISA) of the cultivation supernatants.

With the exception of IF labeling, these experiments were repeated using the permanent K4IM cell line in order to compare the response of these cells to the one of human SF in cell culture and to establish them as a substitute in future experiments. The obtained results could be used to characterize the interplay between the pro-inflammatory TNFα and the anti-inflammatory IL-10, which shed more light on the pathogenesis of OA. The following figure represents the methodical approaches of this study [Fig. 1.6].

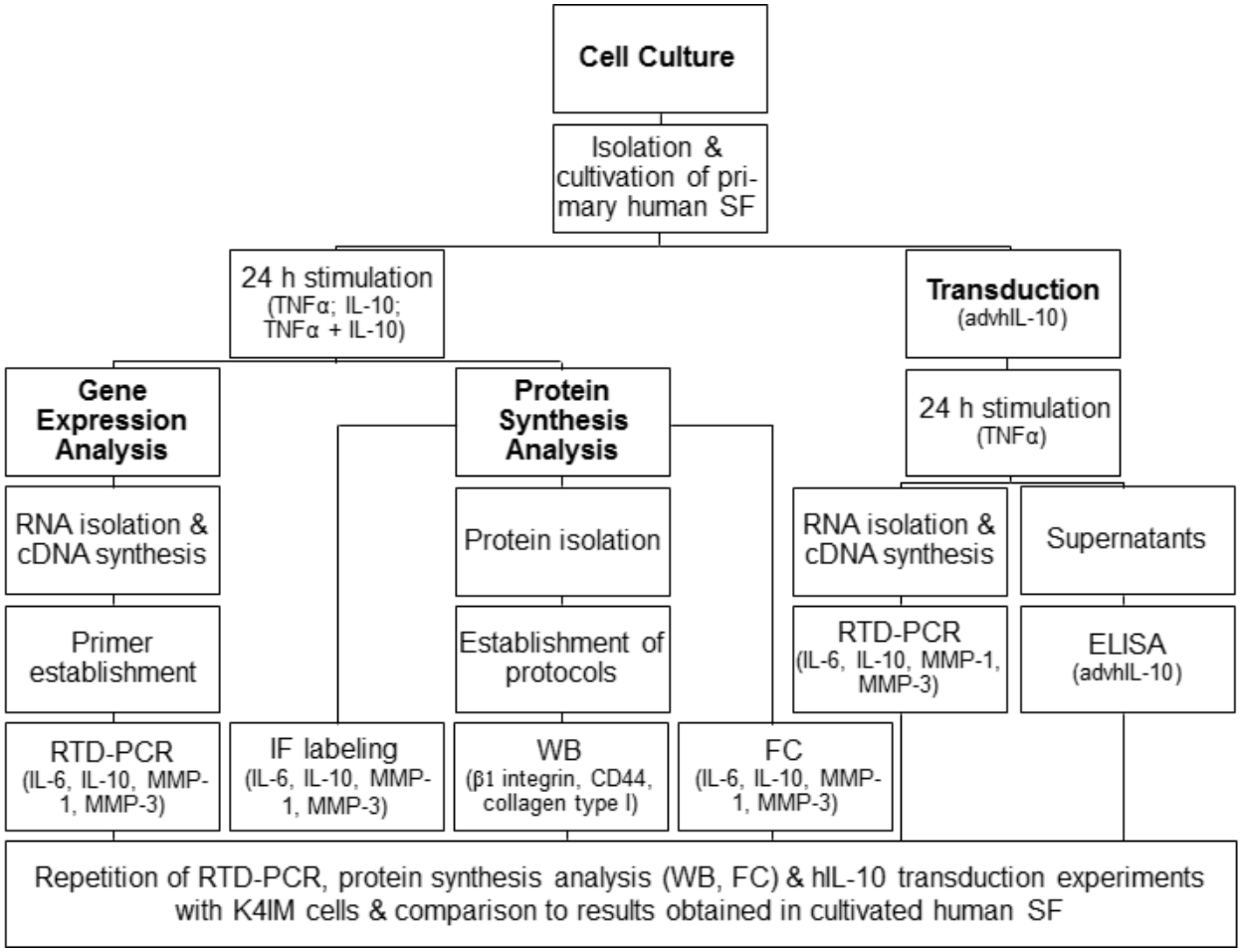


Fig. 1.6: Flow chart detailing the experiments on human SF in cell culture and K4IM cells

2. Materials & Methods

2.1 Materials

2.1.1 Equipment

Piece of equipment	Manufacturer
AF 100	Scotsman, Italy
Biofuge fresco	Heraeus Holding GmbH, Germany
Bio-Rad Model 680 Microplate Reader	Bio-Rad Laboratories, Inc., USA
Centrifuge 5415 D	Eppendorf AG, Germany
Centrifuge & Vortex	neoLab®, Germany
Columbus washer 1.3	Tecan Group Ltd., Switzerland
Counting chamber "Neubauer Improved"	LaborOptik, Germany
Cryo – Safe™ Coolers	neoLab®, Germany
DNA Engine OPTICON™ I	MJ Research, USA
Double PCR Workstation UVT-S-AR	Grant Instruments, USA
FACS Calibur	BD Biosciences, USA
Fluorescence microscope Axioskop 40	Carl Zeiss AG, Germany
Freezer HERAfreeze HFU 686 Basic (-80 °C)	Heraeus Holding GmbH, Germany
Freezing gradient container	Thermo Scientific Nalgene, USA
GelDoc 2000	Bio-Rad Laboratories, Inc., USA
IKAMAG RCT Magnetic Stirrer	IKA®-Werke GmbH & Co. KG, Germany
Incubator Heracell 150; 37 °C, 5 % CO_2	Thermo Fisher Scientific Inc., USA
Invert microscope Axiovert 25	Carl Zeiss AG, Germany
IKA® MS 1 shaker	SIGMA-Aldrich, Germany
Mastercycler® PCR Cycler	Eppendorf AG, Germany
Microplate Reader GENios Tecan	Trading AG, Switzerland
Microscope camera Olympus XC 30	Olympus Soft Imaging Solution GmbH, Germany
Mini-PROTEAN 3 electrophoresis cell	Bio-Rad Laboratories, Inc., USA
Mini Trans-Blot® cell	Bio-Rad Laboratories, Inc., USA
Model 680 microplate reader	Bio-Rad Laboratories, Inc., USA
Multifuge 1 S + 1 S-R	Heraeus Holding GmbH, Germany
NanoDrop 1000 spectrophotometer	Peqlab Biotechnologie GmbH, Germany
Perfect Spin P PCR Plate Spinner	Peqlab Biotechnologie GmbH, Germany
Pipettes Eppendorf Reference® (0,5-10/10-100/50-200/100-1000 µL)	Eppendorf AG, Germany
Pipetus®	Hirschmann Laborgeräte, Germany
PowerPac™ HC High-Current Power Supply	Bio-Rad Laboratories Inc., USA
Refrigerator Comfort No Frost	LIEBHERR, Germany
Scanjet G4050	Hewlett Packard, USA
Scout PRO SP402	Ohaus Corp., USA
Shaker KL2	Edmund Bühler Labortechnik, Germany

Simplicity 185 Personal Ultrapure DI water system	Millipore, USA
Sterile benches	Holten LaminAir, Denmark/ Heraeus Holding GmbH, Germany
Techne DRI-Block heater DB-2D	Bibby Scientific Limited**,** UK
Water bath WBU 45	Memmert GmbH & Co. KG, Germany

2.1.2 Consumable materials

Consumable	**Manufacturer**
6-Well Plates	BD Biosciences, USA
96-Well Plates	BD Biosciences, USA
BD Falcon™ tubes 15 mL	BD Labware, USA
Biosphere® filter tips 10/100/1000 µL	SARSTEDT, Germany
Blotting paper	Schleicher & Schuell, Germany
Cannulas (22G 1 1/2) BDMicrolance™3	BD Labware, USA
Cell culture flasks T25/T75/T175	SARSTEDT, Germany
Cover slips 26 x 21 mm	Gerhard Menzel GmbH, Germany
Cover slips (12 mm diameter)	VWR, Germany
Cryotubes	VWR International™, Canada
Disposable scalpel No. 20	Feather Safety Razor Co., Japan
Disposable sterile pipette tips with microcapillary for loading Gels 1-200 µl	VWR, France
Falcon tubes 15/50 mL	SARSTEDT, Germany
Flexible 12 x 8 Tear-Off Tube Strip Mat	BIOplastics BV, Netherlands
Filter paper (90 mm, 516-0814)	VWR, France
Filtropur S 0.45 µm	SARSTEDT, Germany
Gloves Micro-Touch® Nitra-Tex®	Ansell, United Kingdom
High performance chemiluminescence films (Amersham Hyperfilm ECL [18 × 24 cm])	GE Healthcare Limited, United Kingdom
Immuno 96 MicroWell™ Solid Plates	Nunc GmbH & Co. KG, Germany
Microscope slides 76 x 26 mm	R. Langenbrinck Labor- und Medizintechnik, Germany
Opti-Seal Optical Disposable Adhesive	BIOplastics BV, Netherlands
Parafilm®	Pechiney Plastic Packing, USA
Pipettes 5/10/25 mL, Falcon®	BD Labware, USA
Pipette tips (10 µL, 200 µL, 1000 µL)	SARSTEDT, Germany
PVDF membranes (Pore size 0.22 µm)	Millipore Corporation, USA
Quality Tips, 1250 µL, extra long	SARSTEDT, USA
SuperFrost®Plus object slides	R. Langenbrinck, Germany
Syringes BD Discardit™ II	BD Labware USA
Test tubes 1.5/2.0 mL	Eppendorf AG, Germany
Tissue Culture Dish (Ø 35, 60, 100 mm)	SARSTEDT, USA
UV plates 96-well	Corning Incorporated, USA
Weighing dishes	R. Langenbrinck, Germany

2.1.3 Chemicals, mediums and buffers

Chemical or buffer	Manufacturer
1,4-Dithio-DL-threitol (DTT)	Sigma-Aldrich®, Germany
4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES)	Invitrogen, USA
4',6-Diamidino-2-phenylindol (DAPI)	Roche Diagnostics GmbH, Germany
Loading dye solution, 6x	Fermentas International Inc., USA
50 bp DNA ladder	Invitrogen, USA
Acetic acid, 100%	MERCK, Germany
Acrylamide, 30%	Bio-Rad Laboratories Inc., USA
Agarose High Resolution	Carl Roth GmbH & Co KG, Germany
Ammonium peroxodisulphate (APS)	Carl Roth GmbH & Co KG, Germany
Aqua purificata, pyrogen free, hypertonic	Carl Roth GmbH & Co KG, Germany
β-Mercaptoethanol	Carl Roth GmbH & Co KG, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co KG, Germany
Brefeldin A	Cell Signaling Technology Inc., USA
Buffer RW1, RLT, RPE, RDD	Qiagen, Germany
Collagenase®	Serva electrophoresis, Germany
Complete Mini Protease Inhibitor Cocktail	Roche Diagnostics, USA
Coomassie Brilliant Blue	Carl Roth GmbH & Co, KG, Germany
Dimethyl sulfoxide (DMSO)	Serva, Germany
DNase I	Qiagen, Germany
Donkey serum	Chemicon, USA
Dulbecco´s Modified Eagle´s Medium/Ham's (1:1) F-12 (DMEM/Ham)	Biochrom AG, Germany
Dulbecco's Phosphate buffered saline (PBS) 1x,10x Without Ca^{2+}/Mg^{2+}	PAA Laboratories GmbH, Austria
Essential amino acids, 50x MEM-AS	Biochrom AG, Germany
Ethanol 70 %, 80 %, 96 %	MERCK, Germany
Ethidium bromide solution, 1 %	Carl Roth GmbH & Co KG, Germany
Ethylene glycol tetra acetic acid (EGTA)	Carl Roth GmbH & Co KG, Germany
Fetal calf serum (FCS) (heat inactivated 56 °C; 30 min)	Biochrom AG, Germany
Fluoromount G	Southern Biotech, USA
Gene ruler™ ultra low range DNA ladder	Fermentas International Inc., USA
GBX Developer & Replenisher	Kodak, USA
GBX Fixer & Replenisher	Kodak, USA
Glycine	Carl Roth GmbH & Co KG, Germany

Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich®, Germany
HRP Substrate Luminol Reagent	Millipore, USA
HRP Substrate Peroxide Solution	Millipore, USA
Isopropyl alcohol	Carl Roth GmbH & Co KG, Germany
L-ascorbic acid (Vitamin C), 25 mg/mL	Sigma-Aldrich®, Germany
L-glutamine	Biochrom AG, Germany
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich®, Germany
Methanol	Thermo Fisher Scientific Inc., Germany
Na-EDTA	Sigma-Aldrich®, Germany
Nuclease-Free Water	Qiagen, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH & Co KG, Germany
Partricin, 50 µg/mL	Biochrom AG, Germany
Penicillin/Streptomycin, 10.000 U/10 mg/mL	Biochrom AG, Germany
Poly-L-lysine	Biochrom AG, Germany
Precision Plus Protein™ Kaleidoscope Standards	Bio-Rad Laboratories Inc., USA
RNAse Away™	MβP®, Canada
RNAse free water	Qiagen, Germany
Roti®-Block	Carl Roth GmbH & Co KG, Germany
Roti®-Load I	Carl Roth GmbH & Co KG, Germany
Roti®-Load II	Carl Roth GmbH & Co KG, Germany
Roti®-Nanoquant	Carl Roth GmbH & Co KG, Germany
Saponin	Carl Roth GmbH & Co KG, Germany
Sodium azide (NaN_3)	Sigma-Aldrich®, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co KG, Germany
TaqMan® Gene Expression Master Mix	Applied Biosystems (ABI), USA
Tetramethylethylenediamine (TEMED)	Carl Roth GmbH Co KG, Germany
Triton X-100	Sigma-Aldrich®, Germany
TRIZMA® base	Sigma-Aldrich®, Germany
TRIZMA® HCL	Sigma-Aldrich®, Germany
Trypan blue	MERCK, Germany
Trypsin/EDTA Solution,10x (0.5%)	Biochrom AG, Germany
TWEEN®-20	Sigma-Aldrich®, Germany

SF cell culture medium	Percentage of mixture
DMEM/Ham's F-12 [1:1]	85%
FCS	10%
L-glutamine	1%
Essential amino acids	1%
L-ascorbic acid	1%
Partricin	1%
Penicillin/streptomycin	1%

Cryo conservation medium	Percentage of mixture
FCS	90%
DMSO	10%

2.1.4 Recombinant human cytokines

Recombinant cytokine	Manufacturer
Interleukin-10 (IL-10)	PeproTech, Germany
Tumor necrosis factor α (TNFα)	PeproTech, Germany

2.1.5 RTD-PCR Primer

Gene	Primer sequence	Size of amplificate	Manufacturer
β-Actin (BAC) *Homo sapiens*	not released by manufacturer	171bp	ABI, USA
IL-6 *Homo sapiens*	not released by manufacturer	95bp	ABI, USA
IL-10 *Homo sapiens*	not released by manufacturer	74bp	ABI, USA
MMP-1 *Homo sapiens*	not released by manufacturer	133bp	ABI, USA
MMP-3 *Homo sapiens*	not released by manufacturer	98bp	ABI, USA
Tata box binding protein (TBP) *Homo sapiens*	not released by manufacturer	127bp	ABI, USA

2.1.6 Antibodies

Primary antibodies	Size of antigen	Method	Manufacturer
CD29 (β_1 integrin)	130kDa	WB	BD Transduction Laboratories, USA
Collagen type I antibody	95kDa	WB	Acris Antibodies GmbH, Germany
Monoclonal anti-β-actin antibody produced in mouse (Clone AC-15)	42kDa	WB	Sigma Life Science, Germany
CD44 mouse MAB	80kDa	WB	Cell Signaling Technology Inc., USA
Anti-human IL-6 Fluorescin MAB	not released by manufacturer	FC, IF	R & D Systems Inc., USA

(Clone 1936), mouse IgG2B			
Anti-human IL-10 (polyclonal rabbit)	18.6kDa	FC, IF	PEPROTECH, USA
Anti-human MMP-1 PE MAB (Clone 36607), mouse IgG1	not released by manufacturer	FC, IF	R & D Systems Inc., USA
Human-MMP-3, antigen-affinity-purified polyclonal goat IgG	not released by manufacturer	FC, IF	R & D Systems Inc., USA

Isotype controls	**Size of antigen**	**Method**	**Manufacturer**
Mouse IgG1	-	FC, IF	Invitrogen, USA
Mouse IgG2B	-	FC, IF	Invitrogen, USA
Normal goat IgG	-	FC, IF	R & D Systems Inc., USA
Rabbit IgG	-	FC, IF	Invitrogen, USA

Secondary antibodies	**Antigen**	**Method**	**Manufacturer**
Polyclonal goat-anti-mouse Immuno-globulins/HRP	Mouse Ig	WB	Dako Cytomation, Germany
Polyclonal goat-anti-rabbit Immunoglobulins/HRP	Rabbit Ig	WB	Dako Cytomation, Germany
goat-anti-rabbit-FITC	Rabbit Ig	FC	Dianova GmbH, Germany
Donkey F(ab)2 Fragment-anti-goat-APC	Goat Ig	FC	Dianova GmbH, Germany
Alexa Fluor 488® Donkey-anti-rabbit IgG	Rabbit Ig	IF	Invitrogen, USA
Alexa Fluor 488® goat-anti-mouse IgG	Mouse Ig	IF	Invitrogen, USA
Alexa Fluor 488® Donkey-anti-goat IgG	Goat Ig	IF	Invitrogen, USA

2.1.7 Adenoviral transduction vectors

Vector	**Type of vector**	**Method**	**Manufacturer**
Ad5, Code: ZZCB, Lot: 28ABD	Empty vector	Transduction	Canji Inc., USA
Ad5CMV-GFP, Code: GFCB, Lot: 71AAD	GFP vector	Transduction	Canji Inc., USA
Ad5CMV-hIL10, Code: LTCB, Lot: 59AAZ	Human IL-10 overexpression vector	Transduction	Canji Inc., USA

2.1.8 Ready-made systems

Ready-made system	Method	Manufacturer
Human IL-10 ELISA BD OptEIA™	ELISA	BD Biosciences, USA
RNeasy Mini Kit	RNA isolation	Qiagen, Germany
QuantiTec Reverse Transcription Kit	cDNA synthesis	Qiagen, Germany

2.1.9 Software

Software	Method	Manufacturer
Alpha DigiDoc 1201	WB	Alpha Innotech Corporation, USA
CellD Imaging Software	Microscopy	Olympus Europa Holding GmbH, Germany
CellQuest	FC (measurement)	BD Biosciences, Germany
FlowJo	FC (analysis)	Tree Star Inc., USA
GraphPad Prism 5	Statistics	GraphPad Software Inc., USA
Magellan™	ELISA	Tecan Group Ltd., Switzerland
Microplate Manager 5.2.1	Protein isolation, ELISA	Bio-Rad Laboratories, Inc., USA
Nano-Drop ND-1000 V 3.7.0	RNA isolation	Thermo Scientific, USA
Opticon Monitor 3.1	PCR	MJ Research, USA

2.2 Methods

2.2.1 Isolation and cultivation of human SF

Synovial tissue, discarded during joint replacement surgery of OA patients, was used as ancillary sample for cell culturing after approval by the ethics committee of Charité – Universitätsmedizin, Berlin (EA4/054/11). The age of the 6 patients ranged from 64 to 77 years. After extraction, the synovial tissue was stored in sterile phosphate buffered saline (PBS) for maximal 12 hours until cell isolation. The *Lamina synovialis intima* was separated from the other tissue layers of the synovial membrane and sliced into small pieces using a scalpel. Afterwards, it was transferred to cell culture dishes for cultivation with approximately 2ml of SF culture medium. Free floating of the tissue was avoided. The samples were incubated at 37°C, 95% humidity and 5% CO_2 until the primary SF migrated from the explants. The culture medium was changed every 3 to 4 days. After formation of a confluent monolayer, all explants were transferred to a new petri-dish and the adherent cells were passaged for further proliferation. Cultivation of these cells took place in T-25, T-75 and T-175 flasks. The culture medium continued to be changed every 3 to 4 days and

the SF were passaged again as soon as >75% confluency was reached. The cell cultures were checked regularly for contamination, confluency and morphology.

2.2.2 Passaging SF and K4IM cells

The SF were passaged after reaching a confluency of >75%. The culture medium was suctioned. The adherent cells were then rinsed with 1x PBS and incubated with 0.25% trypsin-EDTA solution for 2-3 minutes at 37°C. After visual control of cell detachment under a light microscope, the trypsin-EDTA solution was neutralized with twice as much SF culture medium. The cell suspension was transferred to a 15ml Falcon tube and centrifuged at 400 x *g* for 5 minutes. The supernatant was disposed of and the cell pellet was resuspended in 2ml fresh SF culture medium. After determining the life cell number using a Neubauer improved counting chamber and trypan blue exclusion staining, the cells were seeded in new cell culture flasks at a density of 5 x 10^3 cells/cm^2. Then incubation continued.

2.2.3 Cryogenic conservation and defrosting of SF & K4IM cells

Surplus cells were stored for later experiments. These cells were treated with trypsin-EDTA solution as described in 2.2.2. Then the cell pellet was resuspended in cryogenic conservation medium and transferred to a cryotube. The cryotube was frozen within a freezing box model Cryo-Safe™ at -80°C for 24 hours. Afterwards, it was transferred to a liquid nitrogen tank for further storage.

When cryo-conserved cells were needed for experiments, they were quickly thawed and transferred to a 15ml Falcon tube. 10ml SF culture medium were added to dilute the cytotoxic DMSO and the suspension was centrifuged at 400 x *g* for 5 minutes. The supernatant was disposed of. Afterwards, the cell pellet was resuspended in SF culture medium and the cells were seeded in a cell culture flask for further incubation.

2.2.4 Cytokine stimulation experiments

After reaching sufficient cell numbers and at least passage 5, the human SF in cell culture and K4IM cells were used for cytokine stimulation experiments. One hour prior to the cytokine stimulation, the cells were serum-starved by replacing the normal SF culture medium containing 10% FCS with medium containing only 1% FCS. Then the cells were stimulated with 10ng/ml TNFα, IL-10 or the combination of TNFα + IL-10 in new, serum-reduced (1% FCS) SF culture medium. Non-stimulated, serum-starved cells served as control population. After 24 hours of additional incubation, the RNA was extracted for analysis.

Alternatively, the cells were used for FC analysis (2.2.6), IF staining and photography (2.2.7) or WB analysis (2.2.8).

2.2.5 Gene expression analysis

Gene expression analysis was performed via RTD-PCR. After isolation from the stimulated cells, the RNA concentration and quality were determined. Afterwards, the RNA was reverse-transcribed into complementary DNA (cDNA), which served as template for the RTD-PCR.

2.2.5.1 RNA isolation

The RNA was isolated with the Rneasy-Mini-Kit made by Qiagen according to the manufacturer's instructions. RNA concentrations were determined with the Nanodrop ND-1000 spectrophotometer via absorption measurements at a wavelength of 260nm. One absorption unit at this wavelength equals 40µg of ssRNA per ml. That fact allows for determination of the complete RNA concentration using the following equation:

$$c_{RNA}\,\frac{\mu g}{ml} = A_{260} * 40$$

Additionally, the absorption quotients A_{260}/A_{280} and A_{260}/A_{230} were determined, which should be about 2 for clean RNA without DNA or salt contamination. If contamination of the RNA samples was found to be unacceptably high, they were purified with the Rneasy-Micro-Kit made by Qiagen according to the manufacturer's instructions. Aliquots of the RNA samples were used for cDNA synthesis. The rest was stored in a freezer at -80°C for later usage.

2.2.5.2 Reverse transcription

RNA destined for gene expression analysis was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit made by Qiagen according to the manufacturer's instructions. Aliquots of the cDNA were used for RTD-PCR. The remainder was stored in a freezer at -20°C for later usage.

2.2.5.3 RTD-PCR

Primer establishment

In order to study the stimulation effects on the gene expression of IL-6, IL-10, MMP-1 and MMP-3, the respective primers had to be established first. For this purpose, a screening-PCR with cDNA from non-stimulated and TNFα stimulated SF was performed. The results allowed tentative deductions concerning the relative gene expressions of the target genes in SF. To check if the used primers were targeted at the correct genes and no unspecific by-products were

measured, the cDNA-amplificates were separated using a 3% agarose gel electrophoresis at 80V for 180 minutes: 2µl of the respective PCR-amplificates were mixed with 5µl of loading dye and transferred to gel pockets at the starting point. The two outermost gel pockets were loaded with 5µl DNA-standard each. After electrophoretic separation, the agarose gel was incubated with ethidium bromide solution for 10 minutes and the stained DNA-bands were photographed under UV-light.

Determination of primer efficiency

cDNA from IL-10 stimulated SF was used for the determination of primer efficiencies via a dilution series with four or more steps. A RTD-PCR using the dilution series was performed for each of the primers. The cycle threshold (Ct)-values were determined in triplicates. The predetermined concentrations of cDNA were filled in logarithmically on the x-axis of a Cartesian coordinate system. The mean Ct-values determined for each concentration were filled in on the y-axis. The resulting standard curve allowed for determination of the increment (a) using the following equation:

$$y = a * x + b$$

The determined increments were used to calculate the primer efficiencies (E) as follows (42):

$$E = 10^{(-\frac{1}{a})}$$

RTD-PCR & relative gene expression

The principle of real time detection PCRs (RTD-PCR) is based on fluorescence measurements, which allow the quantification of PCR products in real time. The RTD-PCR probes (TagMan® probes, Applied Biosystems [ABI]) used in this study utilize the so called fluorescence-resonance-energy transfer (FRET) principle: The TaqMan® probe is composed of a specific oligonucleotide sequence with a “reporter” at the 5’ terminus and a “quencher” at the 3’ terminus. As long as the probe remains intact, reporter and quencher remain relatively close to each other and the FRET principle leads to the extinction of any fluorescence signal. When the probe is hydrolyzed by the 5’-3’-exonuclease activity of the Taq-polymerase after hybridization with cDNA, reporter and quencher are separated. A fluorescence signal occurs and can be measured by the thermocycler after each completed PCR cycle. The increase in fluorescence signal strength is directly proportional to the increase in PCR amplificates [Fig. 2.1].

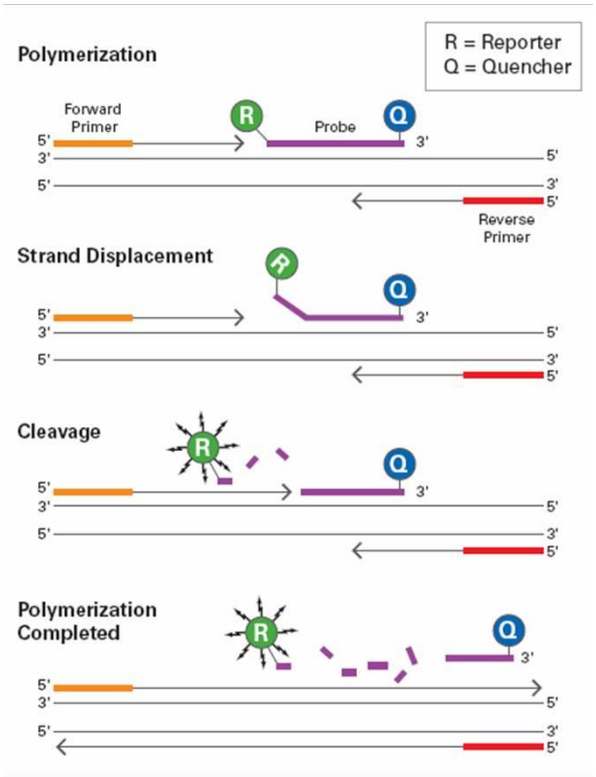


Fig. 2.1: The principle of a TaqMan® RTD-PCR as shown in "TaqMan® Gene Expression Assays Protocol", ABI (2004)

The relative gene expression of IL-6, IL-10, MMP-1 and MMP-3 after cytokine stimulation was determined with RTD-PCRs. β-actin (BAC) in SF and Tata box binding protein (TBP) in K4IM cells, respectively served as points of reference. These housekeeping genes had previously been established in our research group by Ms. Nadine Jork and showed stable gene expression levels in SF (43). Their C(t) values were approximately 23 for BAC with a primer efficiency of 1.90 and approximately 30 for TBP with an efficiency of 1.91 (43). However, BAC expression showed some minor responsiveness to cytokine stimulation in K4IM cells. Therefore, the more stably expressed TBP was used in these cells. The reaction volume measured 20 µl per well. Its composition can be found in table 2.2.

Table 2.2: Composition of the RTD-PCR reaction volume

Component	Volume/Mass
TaqMan® Gene Expression Master Mix	10µl
Primer & probe (all ABI, USA)	1µl
cDNA	16.7ng
RNAse free water	ad 20µl

The relative gene expressions in a single donor were determined in triplicates in a single RTD-PCR run according to the ABI protocol in the thermocycler Opticon I. No template controls ("blanks") with RNAse free water served as negative controls. The PCR protocol can be found in table 2.3.

Table 2.3: RTD-PCR protocol for primers made by ABI

Step	Temperature	Duration	Process
1	50°C	2 minutes	Uracil-DNA glycosylase (UNG) digestion
2	95°C	10 minutes	UNG inactivation & activation of AmpliTaq
3	95°C	15 seconds	Denaturation
4	60°C	1 minute	Hybridization & elongation
5	------	------	Reading the plate
6	------	------	Return to step 3 for 45 more cycles
7	6°C	------	Cooling of amplificates

RTD-PCR analysis was performed with the software Opticon Monitor 3.0. The relative gene expression (R) was determined using the efficiency-corrected method introduced by Pfaffl (42):

$$R = \frac{(E_{\text{target gene}})^{\Delta Ct_{\text{target gene (control-stimulated sample)}}}}{(E_{\text{housekeeping gene}})^{\Delta Ct_{\text{housekeeping gene (control-stimulated sample)}}}}$$

2.2.6 Flow cytometry (FC)

FC was used for the protein synthesis analysis of IL-6, IL-10, MMP-1 and MMP-3. The SF or K4IM cells were stimulated as described in section 2.2.4. Protein release was stopped using 10µg/ml Brefeldin A four hours prior to cell fixation. Brefeldin A inhibits the vesicular transport of proteins from the rough ER to the Golgi apparatus, thereby causing accumulation of proteins within the rough ER (44). Since there was little to no experience with FC in our research group, several different ways of fixation and staining had to be tried out for this study. The method described below was the one that became established for SF and K4IM cells in our research group and was used to obtain the results presented in this study.

FC buffers

The buffers listed below had to be prepared before FC experiments were performed.

Table 2.4.1 FC buffer

Component	Volume/Percentage
BSA	1%
PBS (1x)	ad 500ml
NaN_3	0.01%

Table 2.4.2 Saponin buffer

Component	Volume/Percentage
HBSS	ad 500ml
NaN_3	0.01%
Saponin	0.1%

Cell preparation

After cell harvest as described in 2.2.2, the cell pellet was resuspended in 1ml fresh SF culture medium. The suspension was thoroughly mixed using a pipette and an aliquot of a defined volume was used for cell counting in a Neubauer improved counting chamber. The rest of the cells were centrifuged again at 400 x *g* for 5 minutes and resuspended in ice-cold PBS. Another centrifugation at 400 x *g* for 5 minutes followed. Maximal 5 x 10^5 cells per 0.5ml were resuspended in ice-cold, freshly prepared formaldehyde solution (4%) and incubated at room temperature (RT) for 10 minutes to fix them. Afterwards, the falcon tube was filled with ice-cold PBS and centrifuged again at 400 x *g* for 5 minutes. The supernatant was disposed of, the cells resuspended in ice-cold PBS and centrifuged again as described above.

Antibody immunolabeling, FC measurement and analysis

The cell pellet was resuspended in 1.5ml saponin buffer and the cells were evenly distributed to as many 1.5ml test tubes as necessary for immunolabeling. Afterwards, the test tubes were filled to a volume of approximately 700µl and centrifuged at 400 x *g* for 5 minutes again. The supernatant was disposed of. The cell pellets were resuspended in 250µl of fresh saponin buffer and the primary antibodies were added. Isotype control antibodies served as negative control to rule out non-specific bonds. The tubes were vortexed and incubated at RT in total darkness for 45 minutes. The antibodies detecting IL-6 and MMP-1 had already been coupled with fluorescent stains. Therefore, the cells destined for detection of these antigens only had to be washed twice with saponin buffer as described above before they were resuspended in FC buffer for FC measurements. For IL-10 and MMP-3 detection, the respective test tubes were washed twice with saponin buffer as described above and after disposing of the supernatant the secondary antibodies were added and incubated at RT in total darkness for 30 minutes. When the incubation period was completed, the test tubes were filled to a volume of 700µl and centrifuged

again as described above. The supernatant was disposed of and the cells were resuspended in 700µl FC buffer. After another centrifugation the cell pellets were resuspended in 400µl FC buffer and the cells were transferred to FACS tubes for measurement. The tubes were transported on ice in total darkness until the measurements were taken with the FACS Calibur and CellQuest software. Afterwards, FC analysis was performed using the FlowJo software and the relative protein synthesis compared to the non-stimulated control was determined.

2.2.7 Immunofluorescence labeling

IF buffers

The buffers listed in the tables below had to be prepared before IF staining could begin.

Table 2.5.1 IF blocking buffer

Component	Percentage
Donkey serum	5%
TRIS-buffered salt solution (TBS, 1x)	94%
Triton X-100	1%

Table 2.5.2 10 x TRIS-buffered salt solution (TBS)

Component	Mass/Volume
Aqua dest.	ad 1000ml + pH 7.6
NaCl	87.7g (=1.5M)
TRIS-HCl	78.8g (=0.5M)

Table 2.5.3 1 x TBS

Component	Volume
Aqua dest.	ad 1000ml
NaCl, 1.5M	100ml
TRIS-HCl, 0.5M	100ml

Coating cover slips with poly-L-lysine

The cover slips (12mm diameter) were cleaned with 70% ethanol and autoclaved. Afterwards, they were washed with sterile PBS three times and coated with 10µg/ml poly-L-lysine solution. After 15 minutes of incubation at RT, the cover slips were washed three times with sterile water and dried in an incubator at 37°C. The coated slips were stored at 4°C in a refrigerator until usage.

Colonization, stimulation and fixation of coated cover slips

The coated cover slips were transferred to 6-well plates for colonization and the wells were filled with approximately 2ml SF culture medium containing human SF. After 72 hours of cultivation, the cells were stimulated with TNFα, IL-10 or the combination of TNFα + IL-10 for 24 hours as

described above. Non-stimulated cells served as control group. Four hours prior to cell fixation, intracellular vesicle transport was stopped using 10µg/ml Brefeldin A. The cover slips were washed three times with sterile PBS and fixed in freshly prepared formaldehyde solution (4%) at RT for 15 minutes. Afterwards, they were washed three times with sterile PBS again and stored in sterile PBS at 4°C for later usage.

Labeling fixed human SF on cover slips

The cover slips were rinsed with TBS twice. Then they were incubated with IF blocking buffer for 20 minutes to saturate any unspecific bonds and make the cell membranes permeable for the antibodies. The IF blocking buffer was suctioned and approximately 150µl of primary antibody per cover slip, diluted in IF blocking buffer, was added. Afterwards, the cover slips were incubated in a humid chamber at RT for 45-60 minutes. Cells incubated with isotype control antibodies served as negative control. The 6-well-plates were covered with parafilm in order to minimize evaporation. When the incubation was finished, the slips were rinsed with TBS three times for 5 minutes each. The TBS was suctioned and the secondary antibodies and DAPI 1:40, diluted in IF blocking buffer, were added. Another incubation period of 45-60 minutes at RT in a humid chamber followed and was performed in total darkness. Afterwards, the cover slips were rinsed with TBS three times for 5 minutes each, transferred onto microscope slides and covered with Fluoromount G. Then they were taken to a refrigerator and stored at 4°C until usage.

IF photography

IF photography with the microscope camera Olympus XC 30 and CellD imaging software was performed within 24 hours of cell staining.

2.2.8 Western blot

Western blot (WB) was used for protein synthesis analysis of type I collagen, β_1 integrin and CD44. The SF or K4IM cells were stimulated as described in 2.2.4, then the all proteins were isolated from the cells and their concentration determined using Bradford's principle (45). Afterwards, they were used for WB.

Protein isolation

All steps were performed on ice. In order to isolate the desired proteins, the cells had to be disintegrated first: The SF culture medium was suctioned. Then the adherent cells were rinsed twice with ice-cold PBS and exposed to approximately 0.5ml lysis buffer for 5 minutes. Meticulous scraping with cell scrapers ensured total cell disintegration and maximum protein yield. The composition of the lysis buffer used can be found in table 2.6.

Table 2.6: Composition of the cell lysis buffer for protein extraction

Component	Molarity/Percentage/Quantity	Volume
cOmplete Mini Protease Inhibitor Cocktail	1	-----
DTT	200mM	100µl
EGTA	0.1M	100µl
HEPES pH 7.5	1M	250µl
$MgCl_2$	0.5M	100µl
Triton X-100	10%	100µl
Ultrapure water	-----	9.35ml

Cell lysis was controlled microscopically. Then the suspension was transferred to a 1.5ml Eppendorf reaction tube and centrifuged at 17,000 x *g* and 4°C for 30 minutes. The supernatant containing the desired proteins was transferred to a new 1.5ml reaction tube and a 30µl aliquot was pipetted into a 0.5ml reaction tube for determination of the protein concentration. The samples were stored at -80°C.

Determination of the protein concentration

The protein concentration was determined according to the manufacturer's instructions using Roti®-Nanoquant.

Sample preparation for WB

The protein solution of each sample was diluted with lysis buffer according to the results obtained during the determination of protein concentrations to reach an equal protein concentration in all samples of a given set. The samples were mixed with Roti-Load (4x) in a relation of 3:1 to reach a target volume of 35µl in 1.5ml test tubes. Afterwards, they were denaturized by incubation at 95°C for 5 minutes. Then the samples could be used for WB directly or stored at -20°C until needed.

WB buffers

All the buffers listed below had to be prepared before WB analysis could be performed.

Table 2.7.1 Blocking buffer

Component	Parts
Roti-Block	1
Distilled water	9

Table 2.7.2 Collecting gel buffer

Component	Volume/Mass
Distilled water	ad 500ml
SDS	1g

TRIZMA-base	38.5g
TRIZMA-HCL	9.3g

Table 2.7.3 Running buffer (10x)

Component	Mass/Volume
Distilled water	ad 1000ml
Glycine	144.2g
SDS	10g
TRIZMA-base	30g

Table 2.7.4 Separating gel buffer

Component	Volume/Mass
Distilled water	ad 500ml
HCL	ad pH 6.8
SDS	1g
TRIZMA-base	19.7g

Table 2.7.5 Transfer buffer (10x)

Component	Volume/Mass
Distilled water	ad 1000ml
Glycine	11.25g
TRIZMA-base	2.42g

Table 2.7.6 Transfer buffer (1x)

Component	Volume/Mass
Distilled water	ad 1000ml
Methanol	200ml
Transfer buffer (10x)	100ml

Table 2.7.7 Washing buffer

Component	Volume/Mass
PBS (1x)	ad 1000ml
Tween-20	0.5ml

Sodium dodecyl phosphate polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of the protein samples of any given set was achieved via SDS-PAGE. 7.5% and 10% separating gels and 5% collecting gels were used. The composition of these gels can be found in table 2.8.

Table 2.8 Separation and collecting gel compositions for SDS-PAGE

Component	7.5% separation gel	10% separation gel	5% collecting gel
Acrylamide, 30%	2.50ml	3.30ml	0.80ml
APS, 5%	0.10ml	0.10ml	0.05ml
Collecting gel buffer, pH 8.8	-----	-----	2.50ml

Separation gel buffer, pH 6.8	5.00ml	5.00ml	-----
TEMED	0.01ml	0.01ml	0.01ml
Ultrapure water	2.39ml	1.59ml	1.70ml

30µl of a given sample was pipetted into each gel pocket. The pockets to the far left and right were loaded with 10µl Precision Plus Protein™ standard each. Then the proteins were electrophoretically separated at 80V for 30 minutes and 120V for 60-90 minutes.

Protein transfer

The separated proteins were blotted onto a PVDF membrane that had previously been activated in methanol using an electric field as described in Fig. 2.2.

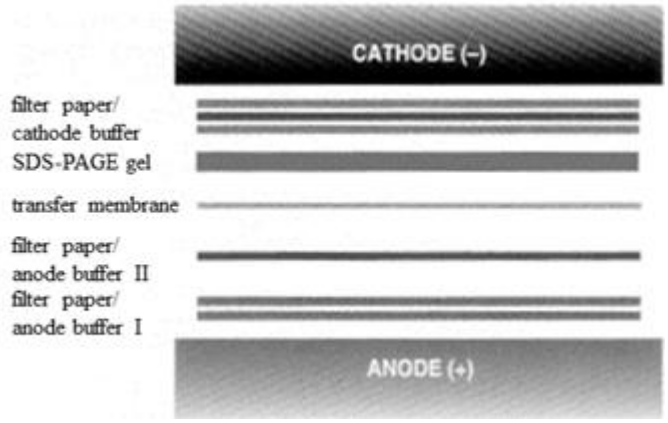


Fig 2.2: Schematic configuration of a blotting chamber
The polyacrylamide gel had to be placed above the PVDF transfer membrane avoiding any air bubbles. Then two filter papers were placed above and below and this "sandwich" was covered with one transfer-buffer-soaked sponge on each side. Finally, the package was inserted into a transfer chamber and the proteins were blotted at 120V for 90 minutes.

Blocking

The PVDF membrane was incubated with blocking buffer at RT for 2 hours to saturate any unspecific binding sites.

Incubation with antibodies, chemiluminescence and densitometric analysis

The PVDF membranes were used to analyze the synthesis of CD44, type I collagen and β_1 integrin. Despite some responsiveness of BAC expression to cytokine stimulation, which had prevented its use for K4IM cells in RTD-PCR, BAC served as a reference protein for both cell populations, since an influence on this housekeeper's protein synthesis could not be detected in initial WB analysis.

The membranes were incubated with the primary antibodies at RT for 2 hours or at 4°C overnight. After washing the membranes with washing buffer, the enzyme-coupled secondary antibody was added and the membranes were incubated at RT for another 2 hours. They were washed again. Then the chemiluminescence reaction was initiated with horse radish peroxidase (HRP) substrate peroxide solution and luminol reagent. The resulting chemiluminescence was detected with high performance chemiluminescence films. Before using the membrane for

detection of the next antigen, it was rinsed in transfer buffer for 30 minutes and washed with washing buffer. The densitometric analysis was performed with the AlphaDigiDoc 1201 software.

2.2.9 Adenoviral transduction

Preliminary test

The adenoviral transduction had already been shown to work in primary and cultured human chondrocytes and cultured SF by other members of our research group. However, a sample of cultured human SF was still seeded onto poly-L-lysine coated cover slides and transduced using 5,000 adenoviral green fluorescent protein (GFP) vector particles per cell. After 24 hours of incubation in SF culture medium containing 1% FCS to allow for full gene expression and protein synthesis, the cells were counterstained with DAPI. Then they were photographed under the microscope camera Olympus XC 30 to illustrate a successful adenoviral transduction for this study.

Cell harvest and seeding for the main experiment

The cells were harvested as described in section 2.2.2. Then the cell numbers were determined using a Neubauer-improved counting chamber and the cells were seeded onto 6-well plates as shown in figure 2.3.

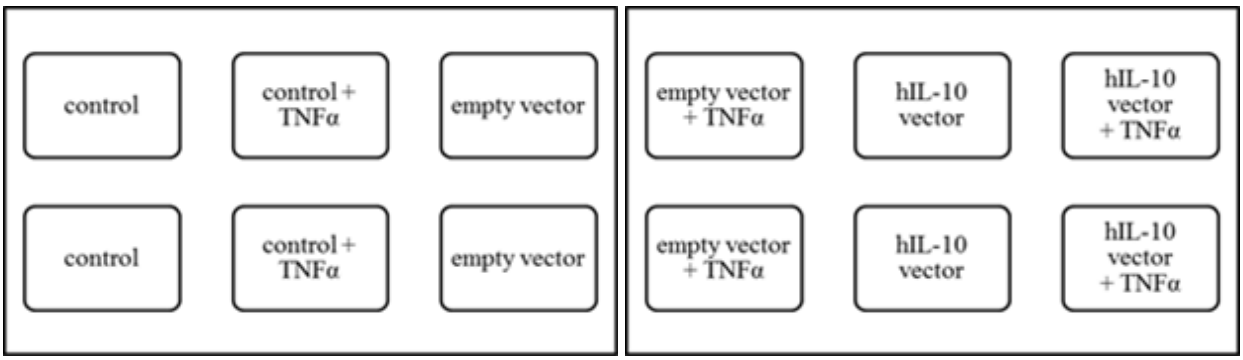


Figure 2.3: Layout for 6-well plates used in the adenoviral transduction experiments
The wells were each seeded with 150,000 cells and treated as follows: The cells in the "control" labeled wells were not transduced. Cells in the wells labeled with "empty vector" and "hIL-10 vector" were transduced with 5,000 empty vector particles per cell or 5,000 adenoviral particles per cell containing the human IL-10 gene. Wells labeled "+ TNFα" were additionally treated with 10ng/ml of TNFα for 24 hours.

An additional cell culture dish with the same area as a single well of a 6-well plate was seeded with 150,000 cells for later determination of cell numbers. After seeding, the cells were incubated for 24 hours with 2ml of SF culture medium (10% FCS) as described earlier. Then the cells in the culture dish were harvested using trypsin-EDTA and counted in a Neubauer-improved counting chamber.

The needed volume of virus particles per well was calculated according to the determined cell numbers.

Adenoviral transduction

The SF were transduced with 5,000 adenoviral vectors per cell. The virus vectors were thawed on ice and the necessary amount of vectors was added to 1ml of serum-reduced SF culture medium (1% FCS). The solution was vortexed and pipetted onto the SF within the 6-wells according to the figures provided above. Non-transduced cells served as control. Afterwards, the 6-well plates were centrifuged at 400 x *g* and 22°C for 5 minutes followed by 4 hours of incubation. The SF were checked microscopically for cytotoxic effects. Then the virus-containing SF culture medium was suctioned and replaced with 1.5ml normal serum-reduced SF culture medium (1% FCS). Another 24 hours of incubation followed to allow full gene expression. Afterwards, the SF culture medium was replaced by new serum-reduced medium. 10ng/ml TNFα was added to the culture medium destined for the wells labeled "+ TNFα". After 24 hours of incubation, the supernatants were transferred to 0.5ml test tubes and frozen at -80°C for later analysis of cytokine release with an IL-10 ELISA. The RNA was isolated from the cells as described in section 2.25 for gene expression analysis with RTD-PCR.

Gene expression analysis

The relative gene expression of IL-6, IL-10, MMP-1 and MMP-3 was determined using RTD-PCR as described in 2.2.5.

IL-10 release analysis

The concentration of released IL-10 in the SF culture medium was determined using the IL-10 ELISA BD OptEIA™ according to the manufacturer's instructions.

2.3 Statistics

The hIL-10 ELISA results were analyzed using one-way ANOVA with Bonferoni post-test. All other experiments were analyzed using a one sample t-test and the results within a given set were normalized *versus* the non-stimulated control. The threshold for statistical significance was set at $p \leq 0.05$. All results were graphically depicted as the mean and standard error of mean (SEM) (mean ± SEM).

2.4 Guidelines

All experiments in this study were performed in accordance with the guidelines provided by Charité – Universitätsmedizin, Berlin (i.e. “Grundsätze zur Sicherung guter wissenschaftlicher Praxis”).

3. Results

3.1 Primer establishment and agarose gel electrophoresis

To analyze the gene expression in SF under the different stimulation conditions, various primers had to be established first. For this purpose, a screening-PCR using the primers of IL-6, IL-10, MMP-1, MMP-3 and VEGF and the cDNA of TNFα-stimulated as well as non-stimulated SF of two donors was performed. BAC had already been established in our research group for SF and was used as a point of reference. After successful RTD-PCR, the amplificates were separated according to their amount of base pairs (BP) using a 3% agarose gel electrophoresis and then photographed under UV light. All PCR amplificates appeared at the expected BP size. No unspecific by-products, which would have been marked by additional bands, were detected [Fig. 3.1].

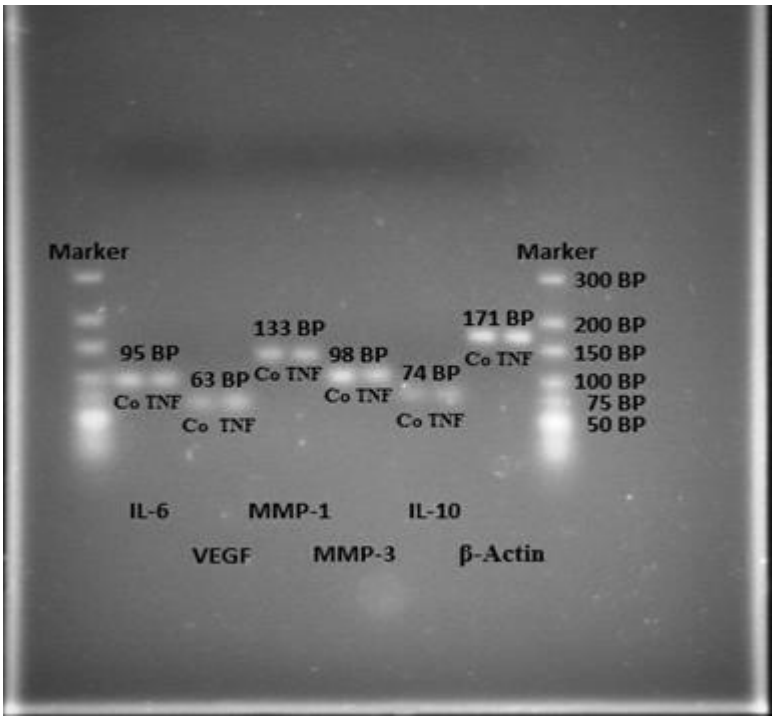


Fig. 3.1: 3% agarose gel electrophoresis
The electrophoretic separation of RTD-PCR amplificates for establishment of the shown primers did not show any unspecific by-products. The respective control bands (Co) can be found on the left while bands of the SF stimulated with 10ng/ml TNFα (TNF) are situated on the right.

3.2 Primer efficiency

The efficiencies of the primers of IL-6, IL-10, MMP-1, MMP-3 and VEGF had to be determined to finalize their establishment. This was done using cDNA dilution series in RTD-PCRs. The C(t)-values of each dilution step were determined in triplicates for every primer. The resulting graphs allowed the determination of their respective increment using the formula $y = a * x + b$. The increment in turn allowed calculation of the primer efficiency using the formula $E = 10^{\frac{-1}{a}}$ according to Pfaffl (42) [Fig. 3.2].

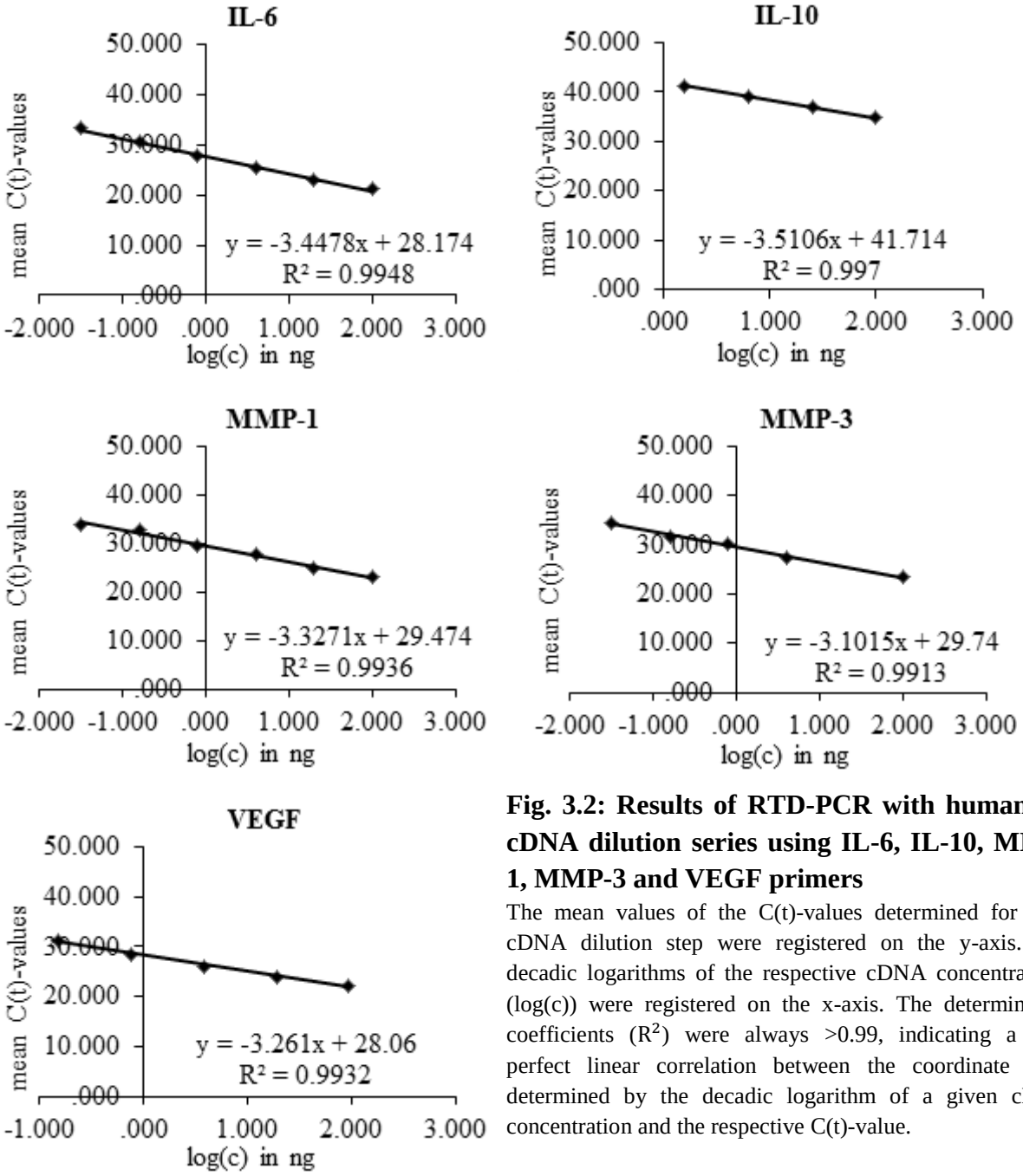


Fig. 3.2: Results of RTD-PCR with human SF cDNA dilution series using IL-6, IL-10, MMP-1, MMP-3 and VEGF primers

The mean values of the C(t)-values determined for each cDNA dilution step were registered on the y-axis. The decadic logarithms of the respective cDNA concentrations (log(c)) were registered on the x-axis. The determination coefficients (R^2) were always >0.99, indicating a near perfect linear correlation between the coordinate pairs determined by the decadic logarithm of a given cDNA concentration and the respective C(t)-value.

Employing the above-mentioned formula, values ranging from 1.93 to 2.10 were calculated for the ABI primers used, indicating very high primer efficiencies [Table 3.1]. However, despite the fact that the results for the primer of VEGF were as good as the rest, it was decided not to perform additional analyses with this gene due to limitations of space on the PCR-plates and limited sample material.

Table 3.1: Calculated primer efficiencies

Primer	Efficiency
IL-6	1.95
IL-10	1.93
MMP-1	2.00
MMP-3	2.10
VEGF	2.03

3.3 Gene expression analysis

3.3.1 Relative gene expression of IL-6

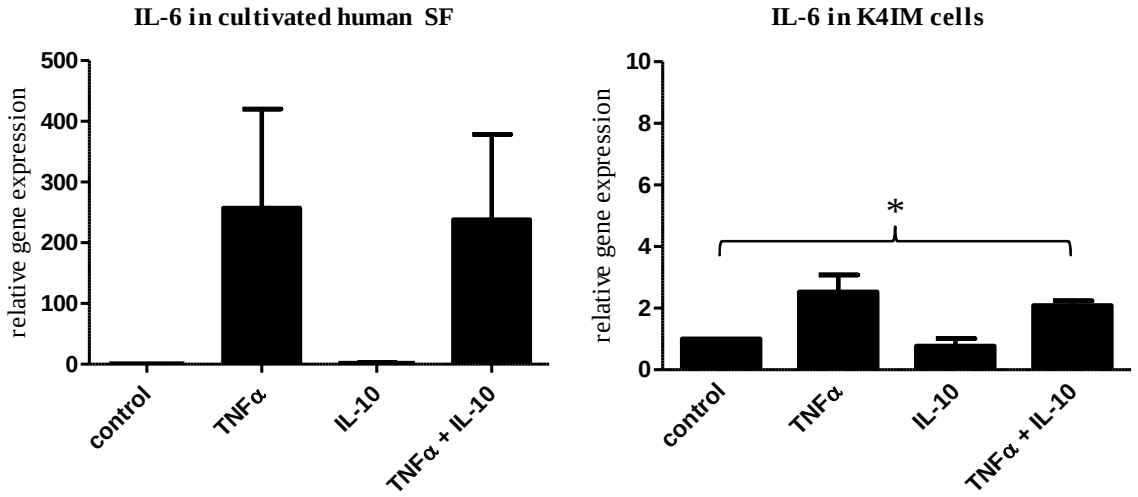


Fig. 3.3.1: Relative gene expression of IL-6 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. The relative gene expression was determined using RTD-PCR. The expression values were normalized according to Pfaffl (42) *versus* the housekeeper BAC in cultured human SF and TBP in K4IM cells, both of which had been previously established in our research group. The column marked with "*" represents a significant result with p=0.0182 in comparison to the control. n=4 for cultured human SF; n=3 for K4IM cells. Please note the very different scaling of the y-axes.

IL-6 was highly up-regulated in TNFα-stimulated and TNFα + IL-10-stimulated samples of human SF in cell culture with a mean expression level that was around 250x higher than that observed in the control group. SF samples receiving the combined treatment showed slightly lower mean gene expression levels than the samples stimulated with TNFα alone. Stimulation with IL-10 caused only a slight elevation in the expression levels of IL-6 in cultured human SF.
In comparison to the control group, the expression levels of IL-6 in K4IM cells were only tripled in TNFα stimulated samples and doubled in samples receiving the combined treatment, which reached statistical significance. Just as in cultured human SF, the stimulation with IL-10 alone had almost no effect on the gene expression levels of IL-6.

Standard deviations were generally much lower in K4IM cells stimulated with TNFα or TNFα + IL-10 when compared to the human SF.

3.3.2 Relative gene expression of IL-10

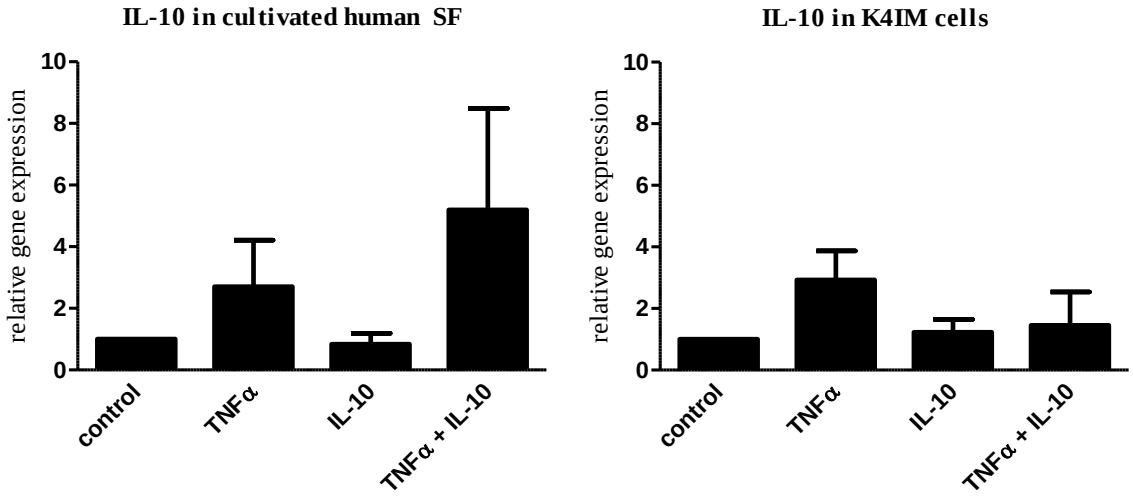


Fig. 3.3.2: Relative gene expression of IL-10 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. The relative gene expression was determined using RTD-PCR. The expression values were normalized according to Pfaffl (42) *versus* the house keeper BAC in cultured human SF and TBP in K4IM cells, both of which had been previously established in our research group. n=4 for cultured human SF; n=3 for K4IM cells.

Stimulation with TNFα caused a detectable increase in the gene expression levels of IL-10 in human SF in cell culture. That effect was even more pronounced in samples stimulated with TNFα + IL-10 and reached roughly a level 5x higher than that observed in the control. Stimulation with IL-10 alone had almost no detectable effect on IL-10 gene expression.
In K4IM cells, stimulation with TNFα or IL-10 had nearly the same effects as described above for cultured human SF. However, the combined treatment did not lead to the highest elevation in the gene expression levels of IL-10 as in human SF in cell culture. Instead, IL-10 expression levels in K4IM cells receiving the combined treatment remained comparable to the control group. Standard deviations in TNFα- or TNFα + IL-10-stimulated K4IM cells were distinctly lower than in cultured human SF again.

3.3.3 Relative gene expression of MMP-1

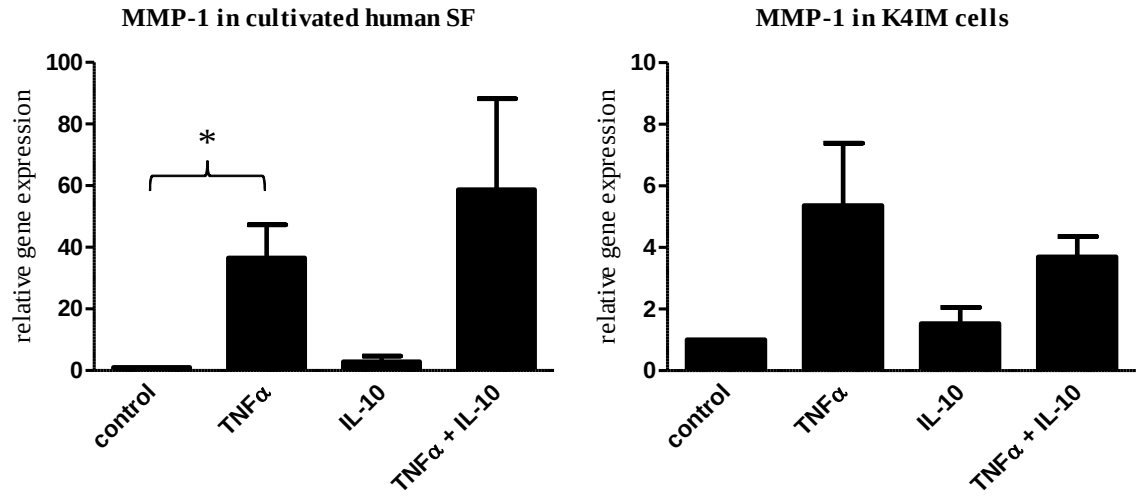


Fig. 3.3.3: Relative gene expression of MMP-1 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. The relative gene expression was determined using RTD-PCR. The expression values were normalized according to Pfaffl (42) *versus* the house keeper BAC in cultured human SF and TBP in K4IM cells, both of which had been previously established in our research group. The column marked with "*" represents a significant result with p=0.0449 in comparison to the control. n=4 for cultured human SF; n=3 for K4IM cells. Please note the very different scaling of the y-axes.

Stimulation with TNFα and TNFα + IL-10 led to a pronounced increase in the gene expression levels of MMP-1 in cultured human SF. TNFα stimulated cells reached levels that were roughly 40x higher, while the combined treatment led to a peak 60x higher than observed control levels. The detected elevation in TNFα stimulated samples was statistically significant. Stimulation with IL-10 alone led to a barely detectable increase in the gene expression levels of MMP-1.

In K4IM cells, stimulation with TNFα or TNFα + IL-10 was also able to increase the gene expression of MMP-1. However, the effects were not as dramatic as in cultured human SF and led only to a 5.5x increase in TNFα stimulated samples and a 4x increase in cells receiving the combined treatment. Interestingly, the effects of TNFα alone on the MMP-1 gene expression in K4IM cells seemed to be stronger than those caused by the combined treatment. Stimulation with IL-10 produced the same effects as in cultured SF.

3.3.4 Relative gene expression of MMP-3

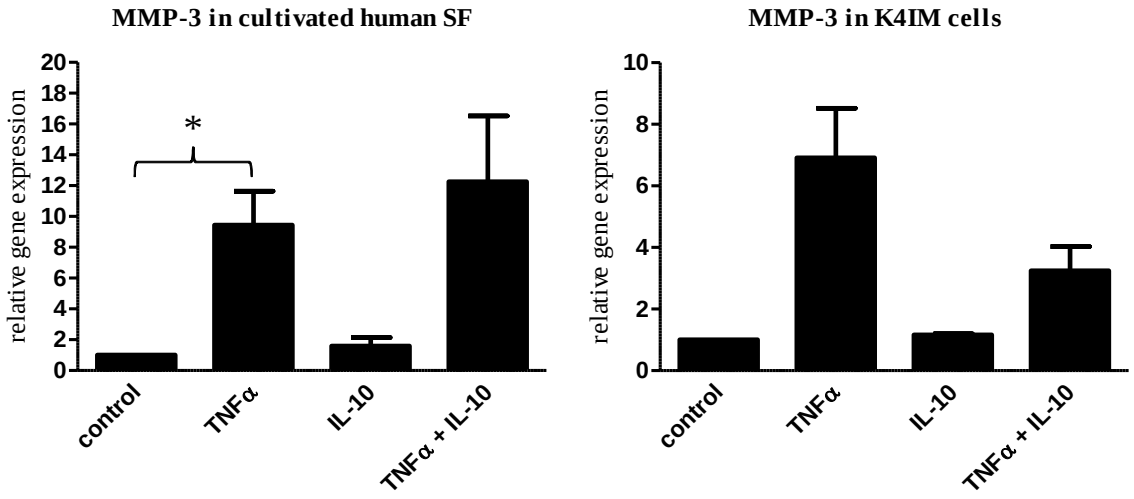


Fig. 3.3.4: Relative gene expression of MMP-3 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. The relative gene expression was determined using RTD-PCR. The expression values were normalized according to Pfaffl (42) *versus* the house keeper BAC in cultured human SF and TBP in K4IM cells, both of which had been previously established in our research group. The column marked with "*" represents a significant result with p=0.0311 in comparison to the control. n=4 for cultured human SF; n=3 for K4IM cells. Please note the different scaling of the y-axes.

In comparison to the control, stimulation with TNFα and TNFα + IL-10 led to a 10x and 12x increase in the gene expression levels of MMP-3 in cultured human SF. The elevation detected in TNFα-stimulated samples was statistically significant. Just as with MMP-1, the stimulation with IL-10 alone led to a barely detectable increase in the gene expression of MMP-3.

When stimulated with TNFα or TNFα + IL-10, the K4IM cells reacted with an elevation in the gene expression levels of MMP-3 that was congruent with the results observed for MMP-1 in K4IM cells. Stimulation with TNFα led to a 7x increase in the gene expression levels of MMP-3, while the combined treatment resulted in a gene expression that was roughly 3x as high as that observed in the control group. Stimulation with IL-10 was unable to elicit an increase in the expression of MMP-3.

3.4 Flow cytometry

3.4.1 Relative protein synthesis of IL-6

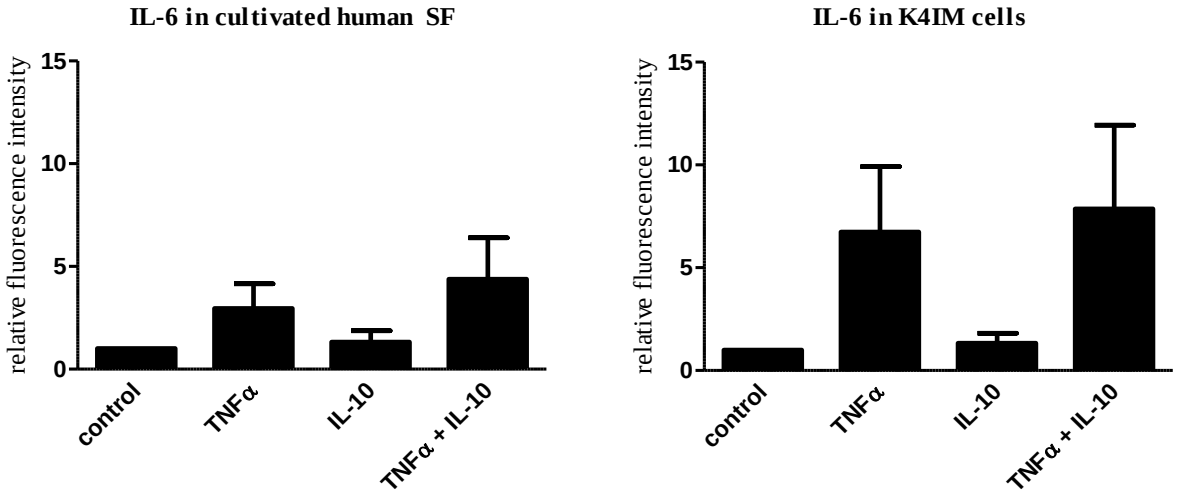


Fig. 3.4.1: Relative protein synthesis of IL-6 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Four hours prior to fixation, intracellular vesicular transport of proteins was arrested with Brefeldin A. Then the cells were harvested, counted, fixed and immunolabeled. Non-stimulated cells served as control. The protein synthesis was measured via relative fluorescence intensity using FC. n=4 for cultured human SF; n=3 for K4IM cells.

The protein synthesis of IL-6 was up-regulated in TNFα-stimulated and TNFα + IL-10-stimulated samples of cultured human SF, reaching a mean fluorescence intensity that was around 4x and 5x higher than that observed in the control. These results differed from those obtained in the RTD-PCR experiments, where induction of the relative gene expression was much higher and the strongest effects were detected in TNFα-stimulated samples. Stimulation with IL-10 caused only a slight elevation in the protein synthesis levels of IL-6 that was comparable to the RTD-PCR findings.

K4IM cells showed the same changes in relative protein synthesis levels as cultured human SF. TNFα and the combined treatment induced a higher relative protein synthesis of IL-6 when compared to the control group and TNFα + IL-10 had the strongest effect. Again, IL-10 alone had almost no effect on the protein synthesis levels. The observed relative protein synthesis levels in K4IM SF were somewhat higher than those found in cultured human SF.

3.4.2 Relative protein synthesis of IL-10

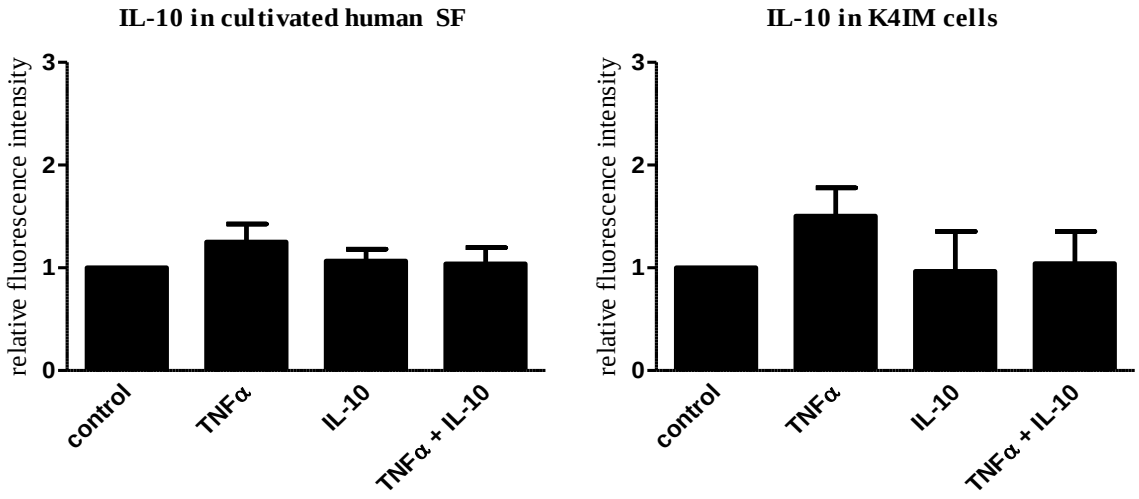


Fig. 3.4.2: Relative protein synthesis of IL-10 in cultured human SF and K4IM cells

The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Four hours prior to fixation, intracellular vesicular transport of proteins was arrested with Brefeldin A. Then the cells were harvested, counted, fixed and immunolabeled. Non-stimulated cells served as control. The protein synthesis was measured via relative fluorescence intensity using FC. n=4 for cultured human SF; n=3 for K4IM cells.

Stimulation with TNFα resulted in a slight elevation in the protein synthesis levels of IL-10 in cultured human SF and K4IM cells. No other stimulation showed effects that could be detected with FC. These results were very different from those observed in RTD-PCR, where stimulation with TNFα and TNFα + IL-10 led to distinctly detectable elevations in the gene expression levels of IL-10, especially in cultured human SF.

3.4.3 Relative protein synthesis of MMP-1

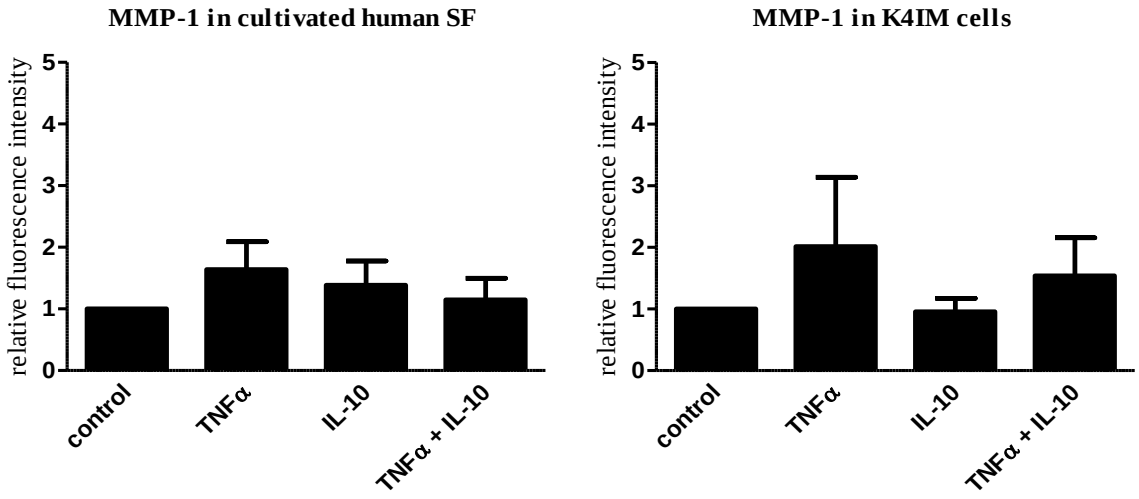


Fig. 3.4.3: Relative protein synthesis of MMP-1 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Four hours prior to fixation, intracellular vesicular transport of proteins was arrested with Brefeldin A. Then the cells were harvested, counted, fixed and immunolabeled. Non-stimulated cells served as control. The protein synthesis was measured via relative fluorescence intensity using FC. n=4 for cultured human SF; n=3 for K4IM cells.

Just as with IL-10, the different stimulations had little effect on the protein synthesis of MMP-1 as detected by FC: In cultured human SF, stimulation with TNFα led only to a slight increase in MMP-1 levels in comparison to the control. The combined treatment had no effects at all and IL-10 caused only a barely detectable increase in MMP-1's protein synthesis as well. These results were very different from those observed in RTD-PCR, where stimulation with TNFα and TNFα + IL-10 led to a clearly detectable increase in the gene expression levels of MMP-1.

Effects were slightly different in K4IM cells. The synthesis of MMP-1 was induced in TNFα-stimulated samples. Additionally, the combined treatment led to some increase in MMP-1's protein synthesis levels. Stimulation with IL-10 had no effect compared to the control. These findings were similar to those observed in RTD-PCR but not nearly as pronounced.

3.4.4 Relative protein synthesis of MMP-3

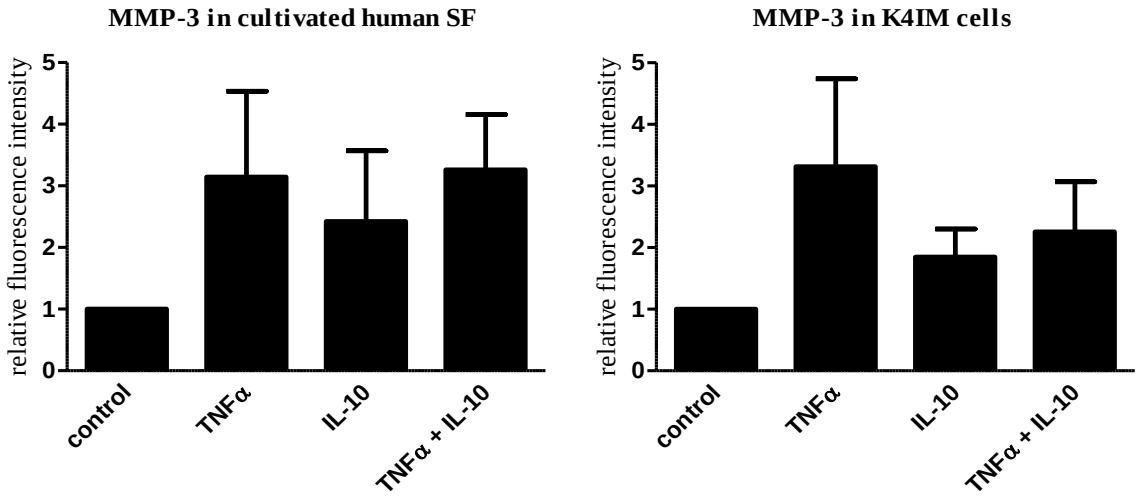


Fig. 3.4.4: Relative protein synthesis of MMP-3 in cultured human SF and K4IM cells
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Four hours prior to fixation, intracellular vesicular transport of proteins was arrested with Brefeldin A. Then the cells were harvested, counted, fixed and immunolabeled. Non-stimulated cells served as control. The protein synthesis was measured via relative fluorescence intensity using FC. n=4 for cultured human SF; n=3 for K4IM cells.

Stimulation with TNFα and TNFα + IL-10 led to an increase in the protein synthesis of MMP-3 in cultured human SF that was around 3x higher than that observed in the control group. Stimulation with IL-10 also led to a 2.5x elevation in detected levels of MMP-3. The K4IM cells showed comparable results. However, the levels of MMP-3 detected in TNFα-stimulated samples were higher than in those receiving the combined treatment.

The results in TNFα- and TNFα + IL-10-stimulated samples of both cell populations were comparable to those observed in RTD-PCR but not as pronounced. Also, stimulation with IL-10 did not induce the gene expression of MMP-3 but apparently influenced its protein synthesis positively. None of the FC results was statistically significant.

3.5 Immunofluorescence labeling

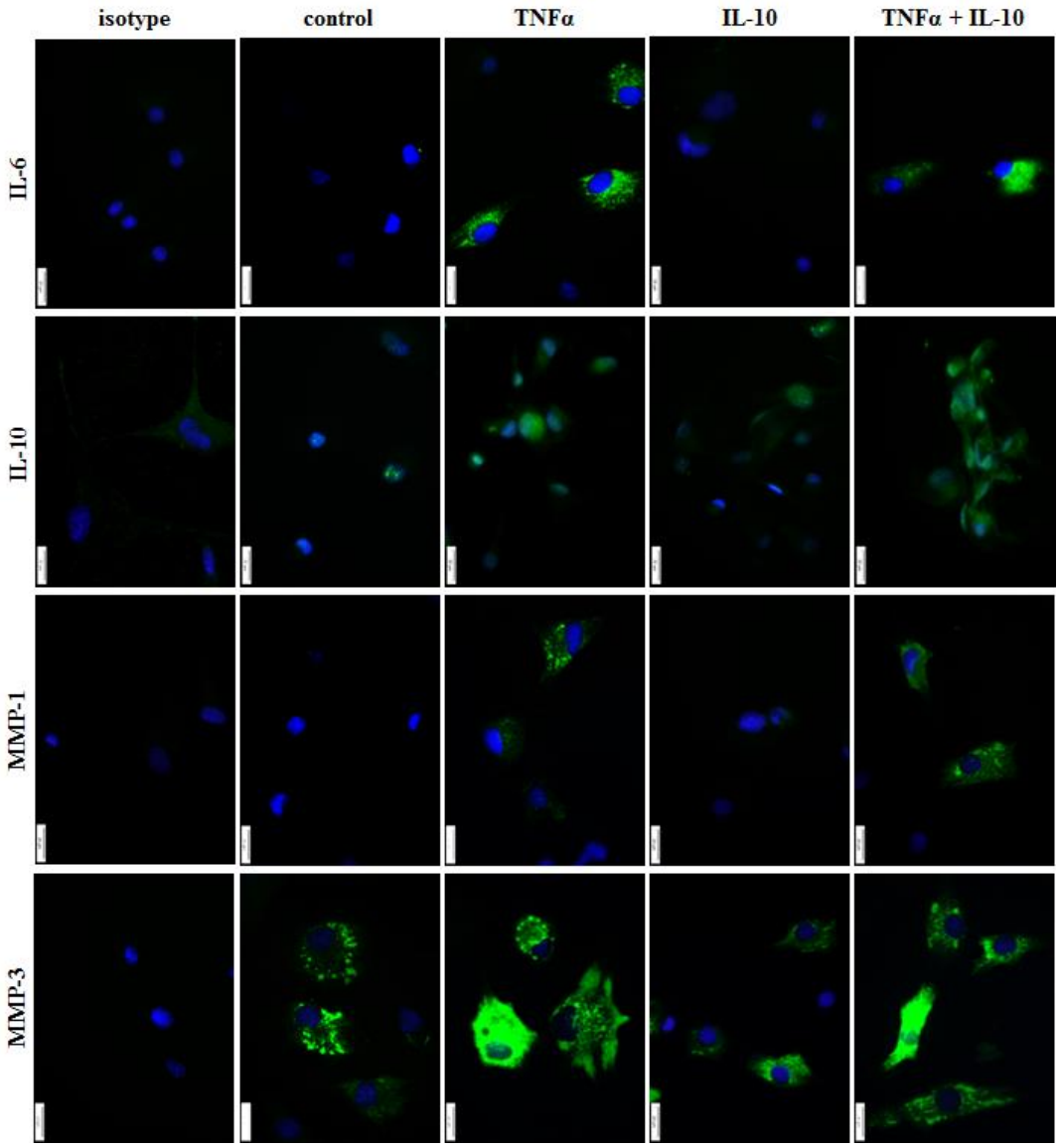


Fig. 3.5: Immunofluorescence-labeled cultured human SF

After 24 hours of stimulation with TNFα, IL-10 or TNFα + IL-10 and blocking of protein secretion with Brefeldin A four hours prior to fixation, cultured human SF of one donor were used for exemplary immunolabeling of the intracellular antigens IL-6, IL-10, MMP-1 and MMP-3. These antigens were stained using primary antibodies and Alexa Fluor 488-labeled secondary antibodies. The cell nuclei were counter-stained using DAPI. Non-stimulated cells served as control. Isotype antibodies of the same antibody class as the respective primary antibodies were used to rule out unspecific bindings. Many but not all cells reacted with an increased protein synthesis of the investigated antigens in response to the stimulations. Fluorescence signals of IL-6, MMP-1 and MMP-3 were restricted to intracellular vacuoles, while the signal of IL-10 was more diffuse. The results were consistent with those obtained using RTD-PCR: Stimulation with TNFα led to the activation of SF and a clearly detectable increase in the amount of every investigated antigen when compared to the control. Stimulation with IL-10 had no effect. TNFα + IL-10-stimulated cells showed an increase in detectable IL-10, MMP-1 and MMP-3, that appeared to be slightly higher than in the sample stimulated with TNFα alone, while IL-6 appeared to be most elevated after stimulation with TNFα only. Scale bar: 20 µm. n=1.

3.6 Western blot

3.6.1 Relative protein synthesis of type I collagen

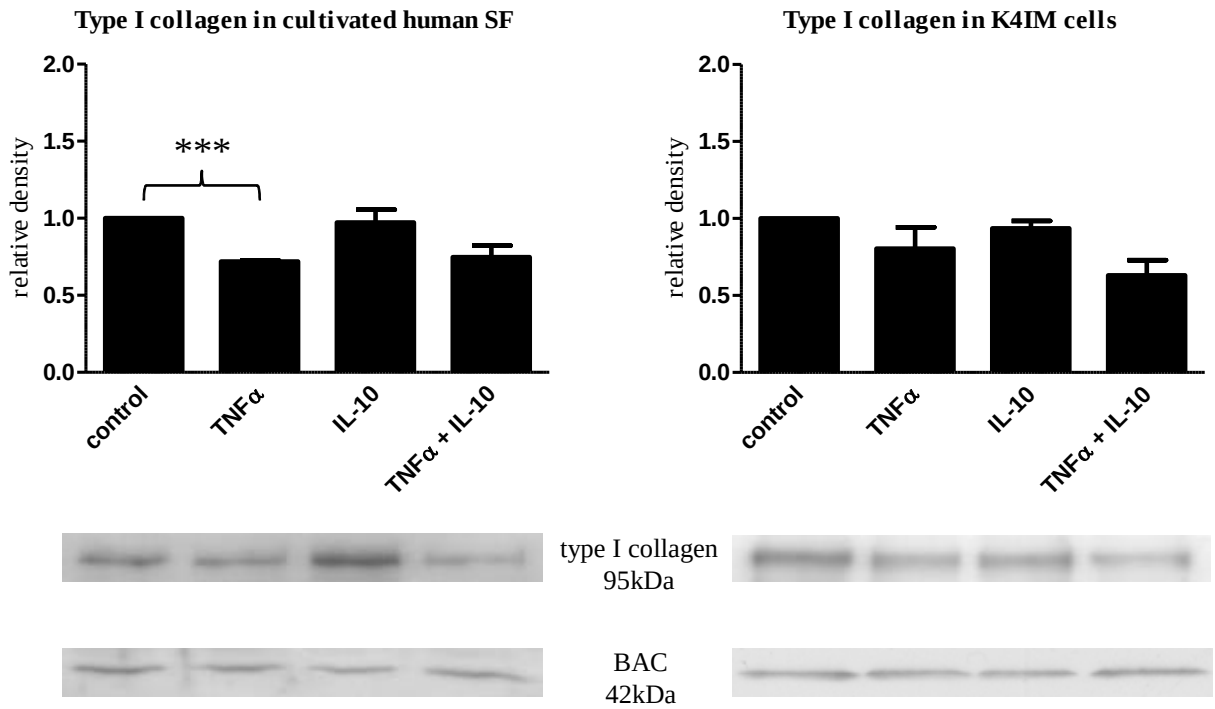


Fig. 3.6: Relative protein synthesis of type I collagen in cultured human SF and K4IM cells

The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. After treatment with Brefeldin A, cell lysis and determination of protein concentrations, WB was performed: The amount of protein utilized in each set was normalized *versus* the control and submitted to SDS-PAGE. Afterwards, the proteins were blotted and the desired antigens were detected using the respective antibodies. The resulting bands were scanned and the relative density compared to the control was determined using Alpha Innotech's Alpha DigiDoc 1201 software. The column marked with "***" represents a highly significant result with p=0.0005 in comparison to the control group. n=3.

The stimulation experiments had the same effects in both cell populations: TNFα led to a decrease in the protein synthesis of type I collagen, which dropped to approximately 0.75x of the amount observed in the control for human SF in cell culture and to 0.8x for K4IM cells. This effect was statistically highly significant in cultured human SF. Stimulation with IL-10 had no effect, while the combination of TNFα + IL-10 led to a decrease in type I collagen concentration, reaching approximately 0.8x of the protein levels observed in the control in cultured human SF and 0.7x in K4IM cells.

3.6.2 Relative protein synthesis of β_1 integrin

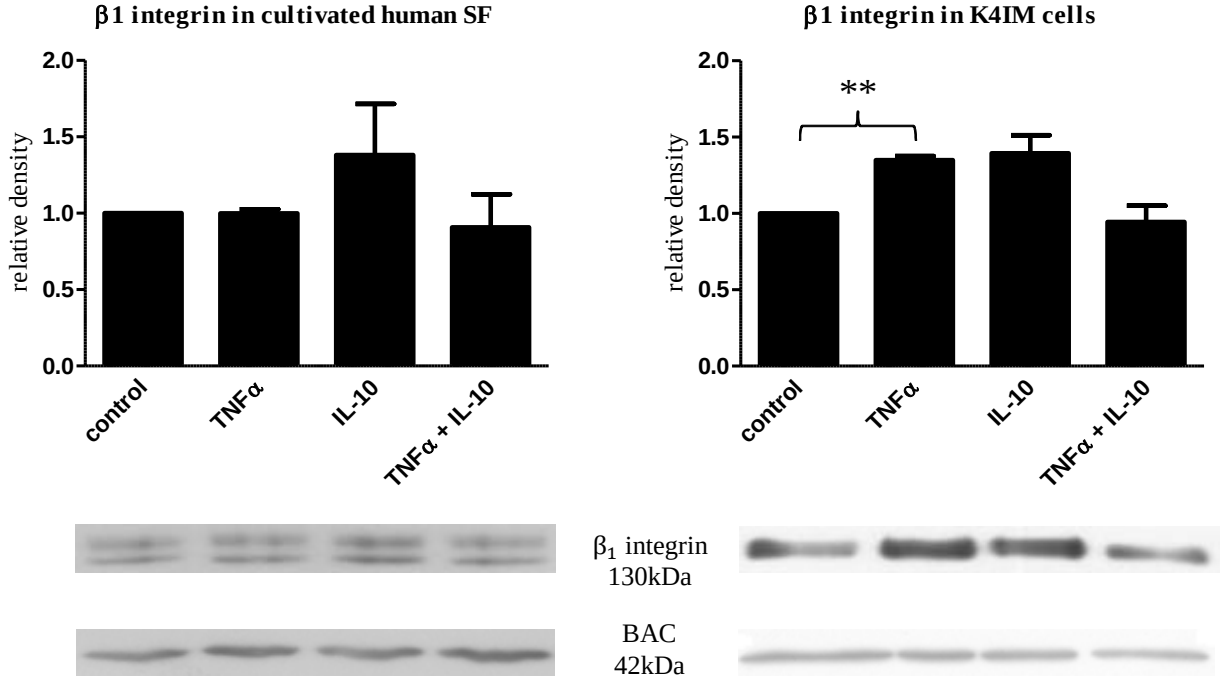


Fig. 3.7: Relative protein synthesis of β_1 integrin in cultured human SF and K4IM cells

The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. After treatment with Brefeldin A, cell lysis and determination of protein concentrations, WB was performed: The amount of protein utilized in each set was normalized *versus* the control and submitted to SDS-PAGE. Afterwards, the proteins were blotted and the desired antigens were detected using the respective antibodies. The resulting bands were scanned and the relative density compared to the control was determined using Alpha Innotech's Alpha DigiDoc 1201 software. Please note that the double bands in the representative blot of cultured human SF were most likely caused by different glycosylation states of β_1 integrin in these cells. The column marked with "**" represents a statistically very significant result with p=0.0059 in comparison to the control. n=3.

As with type I collagen, the stimulation experiments mostly had the same effects in both cultured human SF and K4IM cells. Stimulation with IL-10 induced the protein synthesis of β_1 integrin, leading to a relative density that was approximately 1.4x higher than that of the control, and TNFα + IL-10 caused a slight decrease in the detected protein concentration, which fell to roughly 0.9x that of the control level. The only detectable difference occurred after stimulation with TNFα alone, which seemed to have no effect on the β_1 integrin synthesis in cultured human SF but induced it in K4IM cells, so that the relative density increased to 1.4x that of the control. This observation in K4IM SF was statistically very significant.

3.6.3 Relative protein synthesis of CD44

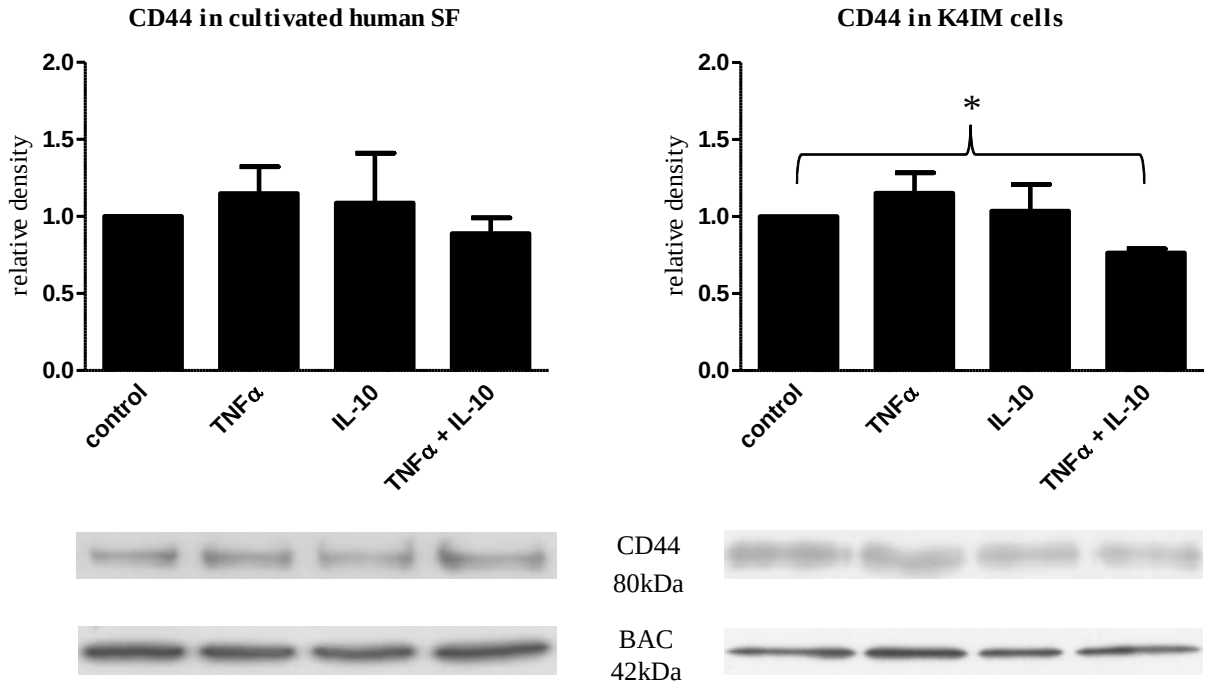


Fig. 3.8: Relative protein synthesis of CD44 in cultured human SF and K4IM cells

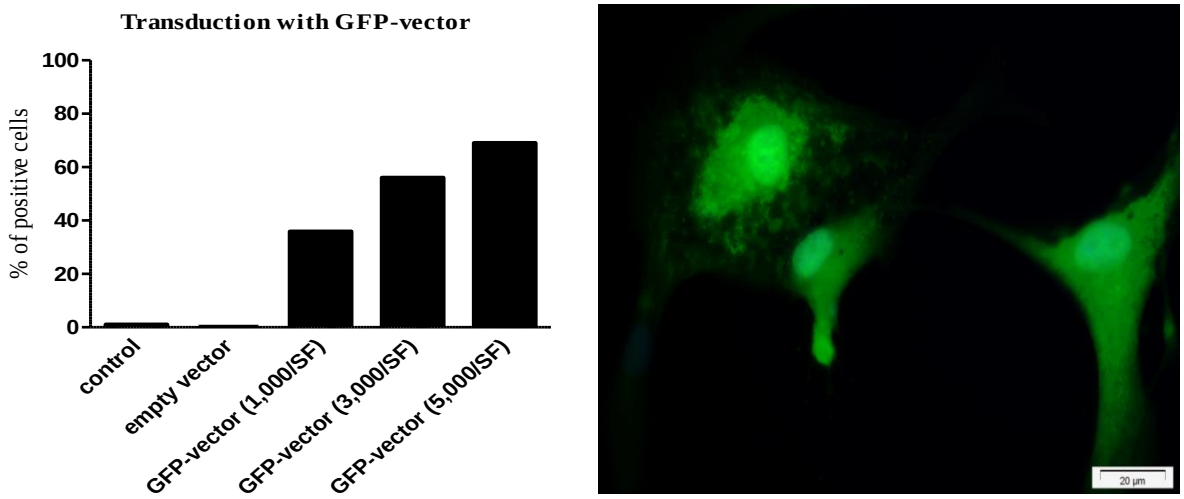
The cells were stimulated with TNFα, IL-10 or with the combination of TNFα + IL-10 at a concentration of 10ng/ml each for 24 hours. Non-stimulated cells served as control. After treatment with Brefeldin A, cell lysis and determination of protein concentrations, WB was performed: The amount of protein utilized in each set was normalized *versus* the control and submitted to SDS-PAGE. Afterwards, the proteins were blotted and the desired antigens were detected using the respective antibodies. The resulting bands were scanned and the relative density compared to the control was determined using Alpha Innotech's Alpha DigiDoc 1201 software. The column marked with "*" represents a statistically significant result with p=0.0124 in comparison to the control. n=3.

Again, the stimulation experiments elicited the same responses in both cultured human SF and K4IM cells: TNFα led to a slight increase in detectable CD44 levels, reaching levels which were 1.2x higher than those in the control. IL-10 had a comparable effect. Stimulation with the combination of both TNFα + IL-10 on the other hand led to a decrease in the detected relative density levels. This effect was not very strong in cultured human SF and only led to a relative density that was 0.9x that of the control, but it was more pronounced in K4IM cells. In comparison to the control, the detected CD44 levels in these samples dropped to roughly 0.75x, which was statistically significant.

3.7 Adenoviral transduction

Cultured human SF were transduced using adenoviral GFP-vector particles to determine the optimal number of adenoviral vector particles per cell in a preliminary test [Fig. 3.9a]. IF pictures were taken to illustrate the successful transduction [Fig 3.9b]. Afterwards, cultured human SF of 3 donors and 3 separate samples of K4IM cells were transduced using either 5,000

empty adenoviral vector particles or 5,000 adenoviral vector particles containing the hIL-10 gene per cell.



a) b)

Fig 3.9: Preliminary transduction of cultured human SF with adenoviral GFP-vector particles

a) The relative number of successfully transduced human SF in cell culture after 24 hours of incubation was directly correlated to the applied number of adenoviral GFP-vector particles per cell. The utilization of 5,000 vector particles per cell achieved the highest transduction rate with about 70 percent of positive cells. Apoptosis was not measured. n =1.

b) These cells were successfully transduced using 5,000 adenoviral GFP-vector particles per cell. Within 24 hours after the transduction, they started to produce the GFP protein in large quantities, as was demonstrated by their distinct green fluorescence. The cell nuclei were stained with DAPI. Scale bar: 20 µm.

3.7.1 Relative gene expression of IL-6 after adenoviral transduction

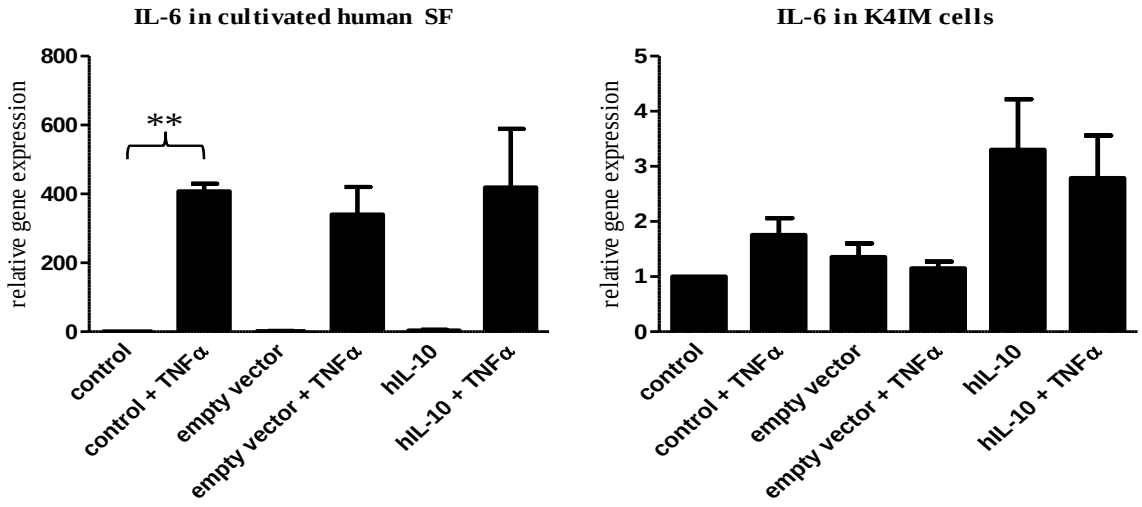


Fig. 4.1: Relative gene expression of IL-6 after adenoviral transduction in cultured human SF and K4IM cells

The cells were transduced using an empty adenoviral vector or a vector containing the hIL-10 gene at a concentration of 5,000 vector particles per cell. After 24 hours of incubation, half the wells were stimulated with TNFα at a concentration of 10ng/ml for an additional 24 hours. Non-transduced cells served as control. Afterwards, cell lysis was induced and the mRNA was extracted. After reverse transcription into cDNA, RTD-PCR was

performed to assess the relative gene expression levels of IL-6 normalized *versus* the control. The column marked with "**" represents a very significant result with p=0.003 in comparison to the control. n=3. Please note the very different scaling of the y-axes.

TNFα strongly induced the mRNA synthesis of IL-6 in cultured human SF. In cells that were only stimulated with TNFα, the gene expression reached levels 400x higher than those in the control group. These results were statistically very significant. SF transduced with the empty vector or the hIL-10 vector and then stimulated with TNFα also increased the mRNA levels of IL-6 to roughly 350x and 400x of that observed in the control group. However, these results were statistically not significant due to a higher distribution width. Neither transduction with the empty vector nor with the hIL-10 vector seemed to influence the gene expression of IL-6 on its own.

The K4IM cells only doubled their expression of IL-6 when stimulated with TNFα, just as in the original stimulation experiments (see section 3.3.1). However, transduction with the hIL-10 vector or empty vector seemed to induce IL-6. That effect was more pronounced in hIL-10-transduced cells and led to a gene expression 3.5x above control level, while transduction with the empty vector only caused a 1.5x higher mRNA concentration. IL-6 was also induced if cells were transduced and stimulated with TNFα. However, the gene expression levels were slightly lower than those observed in only transduced samples. None of the results in K4IM cells showed statistical significance.

3.7.2 Relative gene expression of IL-10 after adenoviral transduction

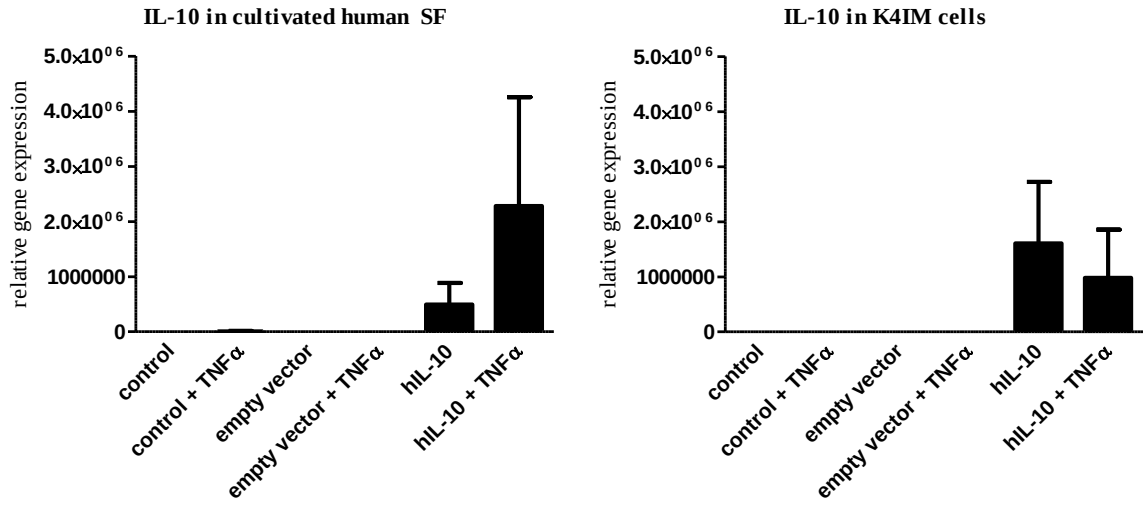


Fig. 4.2: Relative gene expression of IL-10 after adenoviral transduction in cultured human SF and K4IM cells

The cells were transduced using an empty adenoviral vector or a vector containing the hIL-10 gene at a concentration of 5,000 vector particles per cell. After 24 hours of incubation, half the wells were stimulated with TNFα at a concentration of 10ng/ml for an additional 24 hours. Non-transduced cells served as control. Afterwards, cell lysis was induced and the mRNA was extracted.

After reverse transcription into cDNA, RTD-PCR was performed to assess the relative gene expression levels of IL-10 normalized *versus* the control. n=3.

Adenoviral transduction with the hIL-10 gene led to a considerable increase in the mRNA levels of IL-10 in both human SF in cell culture and K4IM cells. In comparison to the control, IL-10 was on average $5 * 10^5$x higher expressed in hIL-10-transduced cultured human SF and even $2.3 * 10^6$x when the cells were additionally stimulated with TNFα. The other treatments did not have a comparable effect, although the stimulation of non-transduced cells with TNFα induced IL-10 slightly, just as it had done in the original stimulation experiments (see 3.3.2). The same effect could be observed in SF that were transduced with empty vectors and then exposed to TNFα.

In K4IM cells, transduction with hIL-10 led to a $1.6 * 10^6$x increase in the mRNA levels of this gene. Cells that were hIL-10-transduced and stimulated with TNFα also showed a pronounced elevation in the expression of IL-10. However, this effect was somewhat weaker and only reached an average increase of approximately $1 * 10^6$x in comparison to the control group. TNFα stimulation of non-transduced SF and K4IM cells that were transduced with the empty vector slightly increased the levels of detected IL-10 mRNA. None of the above-mentioned results was statistically significant due to wide distribution widths.

3.7.3 Relative gene expression of MMP-1 after adenoviral transduction

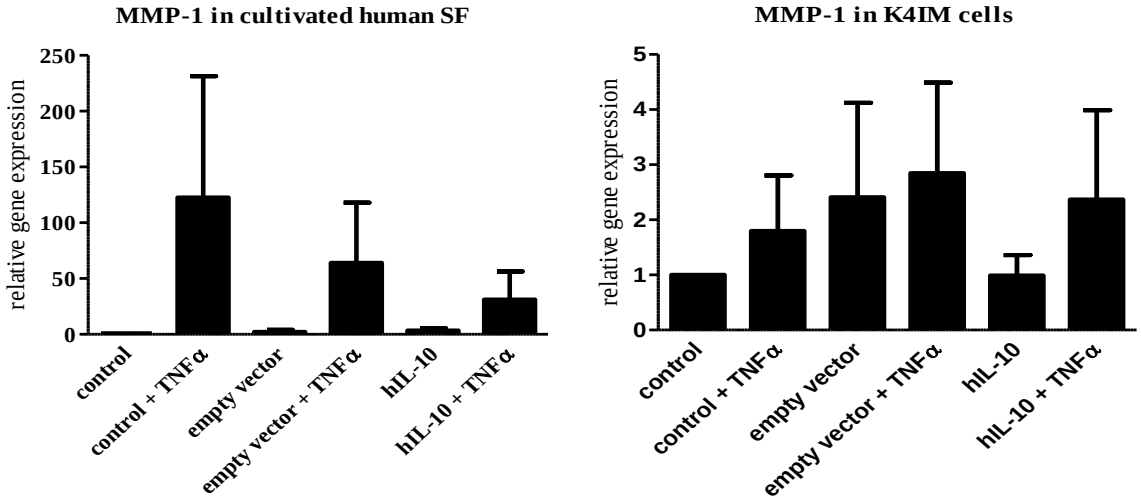


Fig. 4.3: Relative gene expression of MMP-1 after adenoviral transduction in cultured human SF and K4IM cells

The cells were transduced using an empty adenoviral vector or a vector containing the hIL-10 gene at a concentration of 5,000 vector particles per cell. After 24 hours of incubation, half the wells were stimulated with TNFα at a concentration of 10ng/ml for an additional 24 hours. Non-transduced cells served as control. Afterwards, cell lysis was induced and the mRNA was extracted. After reverse transcription, RTD-PCR was performed to assess the relative gene expression levels of MMP-1 normalized *versus* the control. n=3. Please notice the very different scaling of the y-axes.

MMP-1 was highly induced in cultured human SF treated only with TNFα and its gene expression reached levels that were on average 125x higher than those observed in the control. It was also induced in samples transduced with either the empty vector or the hIL-10 vector and then stimulated with TNFα. However, the detected expression levels were not as high and reached only 75x and 25x that of the control group. The transduction with either vector on its own did not seem to have any detectable effect on the mRNA concentration of MMP-1.

In K4IM cells, MMP-1 was also induced by stimulation with TNFα. Just as in the original stimulation experiments, the effects were not nearly as pronounced as in cultured human SF. The gene expression reached only twice the control levels in those cells that were treated with TNFα, 3 x in samples that were transduced with the empty vector and then stimulated and 2.5 x in the K4IM cells that were hIL-10-transduced and treated with TNFα. The process itself seemed to induce MMP-1, when K4IM cells were transduced with the empty vector, and the according mRNA levels detected in these cells were comparable to all the TNFα stimulated samples. However, transduction with hIL-10 without further stimulation did not seem to have any effect. None of the results was statistically significant due to relatively high distribution widths.

3.7.4 Relative gene expression of MMP-3 after adenoviral transduction

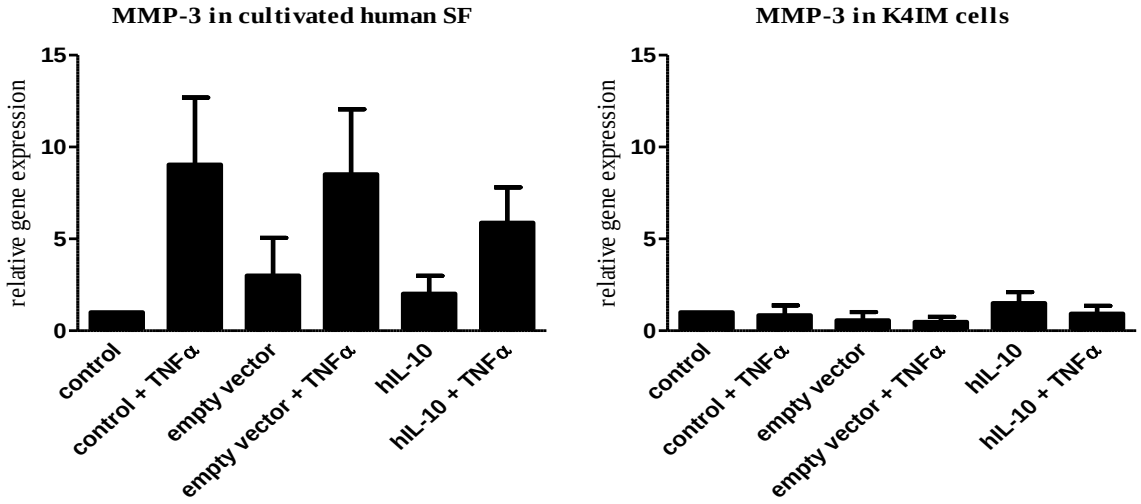


Fig. 4.4: Relative gene expression of MMP-3 after adenoviral transduction in cultured human SF and K4IM cells

The cells were transduced using an empty adenoviral vector or a vector containing the hIL-10 gene at a concentration of 5,000 vector particles per cell. After 24 hours of incubation, half the wells were stimulated with TNFα at a concentration of 10ng/ml for an additional 24 hours. Non-transduced cells served as control. Afterwards, cell lysis was induced and the mRNA was extracted. After reverse transcription, RTD-PCR was performed to assess the relative gene expression levels of MMP-3 normalized *versus* the control. n=3.

As in the original stimulation experiments, MMP-3 was induced in cultured human SF that were not transduced but treated with TNFα and its gene expression reached levels 9x higher than those

observed in the control. The expression of MMP-3 was also increased in samples that were transduced with either the empty or the hIL-10 vector and then TNFα-stimulated. However, effects in these cells were slightly lower and the mRNA levels reached only 8x and 6x that of the control. The transduction process with either vector on their own apparently activated the cultured human SF and prompted them to slightly increase their gene expression of MMP-3, causing it to triple in those cells transduced with empty vectors and double in the ones transduced with hIL-10.

In K4IM cells, MMP-3 appeared to be quite unaffected by transduction, stimulation with TNFα or the combination of both. Its mean expression levels only slightly increased in the hIL-10 transduced samples. None of the results was statistically significant.

3.7.5 IL-10 ELISA

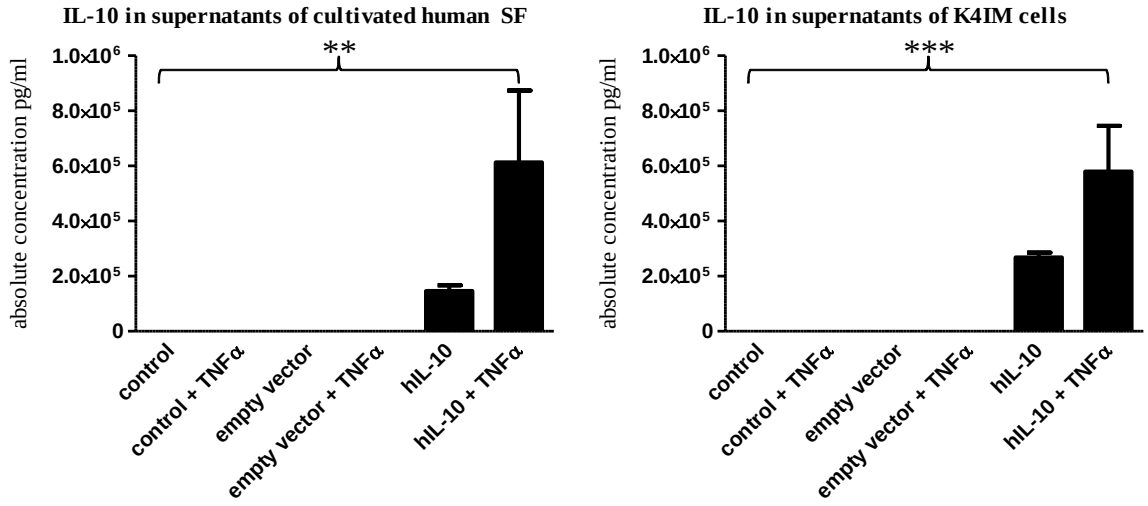


Fig. 4.5: Absolute protein concentration of IL-10 in supernatants after adenoviral transduction of cultured human SF and K4IM cells

The cells were transduced using an empty adenoviral vector or a vector containing the hIL-10 gene at a concentration of 5,000 vector particles per cell. After 24 hours of incubation, half the wells were stimulated with TNFα at a concentration of 10ng/ml for an additional 24 hours. Non-transduced cells served as control. The stimulation supernatants were collected to perform a hIL-10 ELISA to assess the absolute IL-10 concentration after adenoviral transduction. The column marked with "**" represents a statistically very significant result with p=0.0086 and the column marked with "***" represents a statistically highly significant result with p=0.0002 in comparison to the control. n=3.

Adenoviral transduction with the hIL-10 gene led to a considerable increase in the absolute protein concentration of IL-10 in the supernatants of both cell populations: In comparison to the control, hIL-10-transduced cultured human SF produced $1.75 * 10^5$x more IL-10 protein and after additional stimulation with TNFα even $6 * 10^5$x more. The latter increase was statistically very significant when compared to all other absolute IL-10 concentrations in this set. Stimulation with TNFα led to a slight increase in IL-10 protein levels in the supernatants of non-transduced

cultured human SF and cells transduced with the empty vector. However, these effects did not show statistical significance.

In K4IM cells, the hIL-10 transduction led to a $3 * 10^5$x increase in detected IL-10 protein levels in comparison to the control. Cells that were hIL-10-transduced and stimulated with TNFα also showed a pronounced elevation in the absolute concentration of IL-10, reaching levels as high as $6 * 10^5$x those of the control. This increase was statistically highly significant when compared to all the other samples in this set. Stimulation with TNFα led to a slight increase in detectable IL-10 protein levels in the supernatants of non-transduced cells and K4IM cells that were transduced with the empty vector. However, these effects were not statistically significant.

4. Discussion

OA is a well-known global disease that has been reported in Egyptian mummies and even dinosaurs and shows an increase in prevalence with the ageing of the world's population (46, 47). It is the most common form of arthritis and currently affects about 33.6% of U.S. citizens older than 65 years (47), a number that is most likely representative for all industrialized countries. However, its pathogenesis is far from being fully understood and currently no cure is available (46), although three different therapeutic strategies are employed: Pharmacological, non-pharmacological and surgical therapies (48). Pharmacological approaches with non-steroidal anti-inflammatory drugs (NSAIDs) or intra-articular glucocorticoid injections focus mostly on pain management and are not without serious adverse events, including gastrointestinal, cardiovascular and renal side effects, which increase in frequency with the patient's age and co-medications (47). Non-pharmacological approaches include physical therapy, low-impact or strengthening exercises, weight loss for overweight patients and articular taping, but in many cases these options do not lead to adequate pain relief (47). Surgical therapy options, including knee replacements, currently appear to have the best outcome in terms of functionality and cost effectiveness (48). However, a significant proportion of patients are still dissatisfied after knee replacement surgery and the full joint function is never regained (48). Therefore, it is of the utmost importance to finally understand the etiology and pathophysiology of OA and to develop new treatment options (46).

Catabolic cytokines like IL-1 and TNFα have been found in the synovia of OA-affected joints and could be responsible for the cartilage destruction (49). In accordance with this cytokine based disease model, it was the main objective of this dissertation to shed more light on the role of SF in the pathogenesis of OA and to characterize the interplay between the pro-inflammatory cytokine TNFα and the anti-inflammatory IL-10 and to test a possible future cytokine treatment *in vitro*. Additionally, cytokine triggered responses in the permanent K4IM cell line, which consists of immortalized SF of a healthy donor, were compared to the responses observed in cultured human SF extracted from the SM of OA-affected donors. This was done to investigate the possibility of using the K4IM cell line as a model in future OA-related *in vitro* experiments in our research group, thereby circumventing the need for access to primary human SF, inter-individual differences and the slow proliferation rates of these cells.

4.1 Cytokine stimulation effects on the gene expression and protein synthesis of IL-6, IL-10, MMP-1 and MMP-3 in cultured human SF and K4IM cells

Since it was found that cytokines and MMPs are elevated in OA and involved in its pathogenesis (46, 49), some of these mediators were further analyzed in both cultured SF and K4IM cells.

4.1.1 Effects on the expression and synthesis of IL-6

Stimulation with TNFα or TNFα + IL-10 induced an elevated gene expression and protein synthesis of IL-6 in both cultured human SF and K4IM cells. Cultured human SF showed a more pronounced response on both mRNA and protein levels. However, the standard deviation was smaller in K4IM cells. IL-10 did not significantly modulate the effects of TNFα on IL-6 in either experimental setting. These findings clearly show that the gene expression and protein synthesis of IL-6 in SF can be regulated in response to cytokine stimulation. This effect is consistent with the description of elevated IL-6 levels in OA affected joints (17) and may be an indicator that SF facilitate joint destruction by reacting to an increase in intra-articular pro-inflammatory cytokine levels with production of even more (pro-inflammatory or ambivalent) cytokines like IL-6. Similar effects have already been shown for the TNFα induced synthesis of TNFα in cultured human SF (43) and TNFα induced synthesis of IL-6 in K4IM cells (50). The transcription factors NF-κB and AP-1 seem to play an important role in both these processes as well as in the induction of MMPs (50). However, the precise role of IL-6 in the pathogenesis of OA remains controversial and there are both scientific works claiming it to be a contributor (17) and inhibitor of OA (17, 18). Further research with regard to which cytokines and mediators exactly are regulated by IL-6 is definitely required, and its interplay with other cytokines, like IL-10, which also shows elevated intra-articular levels in OA, needs to be characterized to fully understand the role of IL-6 in this form of arthritis.

4.1.2 Effects on the expression and synthesis of IL-10

Higher gene expression of IL-10 was induced by stimulation with TNFα or TNFα + IL-10 in cultured human SF and K4IM cells. Standard deviations in K4IM cells were distinctly lower than in human SF in cell culture. These results, although not statistically significant, show that mRNA levels of IL-10 are subject to cytokine influence. The detected effects on the gene expression were in line with current scientific literature, which indicated that similar results were obtained in cultured human chondrocytes and that elevated levels of this anti-inflammatory cytokine can be found in OA affected joints (13, 46, 51).

However, the protein synthesis of IL-10 as detected by FC did not show any significant response

to stimulation with either TNFα, IL-10 or TNFα + IL-10, which might suggest some form of post-transcriptional or post-translational regulation in SF. Additionally, the incubation period with Brefeldin A might have been too short to cause a high enough protein accumulation for proper FC detection or the labeling process might require further optimization. However, longer Brefeldin A exposure would have had severe side effects, especially on cultured human SF.

Since *in vitro* experiments and clinical trials have suggested that an artificial increase in intra-articular IL-10 levels might hold a lot of promise for the treatment of RA (51, 52), that prospect could also be relevant for the future management of OA. Therefore, the regulatory mechanisms of IL-10 and its effects, especially on the regulation of secondary catabolic mediators like MMPs, require further study.

4.1.3 Effects on the expression and synthesis of MMP-1 and MMP-3

Stimulation with TNFα and TNFα + IL-10 led to a pronounced increase in the gene expression levels of MMP-1 & -3 in cultured human SF and K4IM cells. Stimulation with IL-10 alone had no effect on the mRNA levels of either MMP in either cell population. The effects on the protein synthesis as detected by FC were similar, but in comparison to the results obtained in RTD-PCR, they were much weaker and in the case of MMP-1 barely detectable.

Just as with IL-10, this might indicate post-translational and/or post-transcriptional regulation processes or procedural inefficiency during the labeling process, but it could also be caused by the fact that incubation with Brefeldin A started only four hours prior to fixation to avoid adverse effects and was not long enough to cause properly detectable protein retention.

The increase of MMP-1 &-3 expression and synthesis in response to TNFα was in line with current scientific literature describing similar effects in chondrocytes within OA affected joints (11, 19, 53) and K4IM cells (51). However, IL-10 was able to impair the stimulatory effects of TNFα on the gene expression of both MMPs in cultured human chondrocytes (19), an effect that could not be observed in the experiments with cultured human SF but in those with K4IM cells. This might indicate that either the process by which these SF were immortalized or the prolonged exposure of the cultured human SF to the intra-articular OA cytokine cocktail - they were extracted from OA-affected joints - could have influenced their response to these catabolic mediators. Additional comparison with the cytokine-triggered expression and synthesis responses in cultured human SF from a healthy donor could answer this question.

4.1.4 Immunofluorescence labeling

Results of the exemplary IF photography of cultured human SF were consistent with those obtained using RTD-PCR. Both these findings clearly differ from the ones observed in FC and

might indicate that the FC experiments require further procedural optimization, e.g. longer incubation periods with Brefeldin A, longer cytokine stimulation periods or the use of directly labeled primary antibodies to minimize manipulation during the labeling process. Also, cells used in the FC experiments were fixed prior to exposure to the antibodies, which might have affected FC results. Therefore, it should be tried to use non-fixed cells to exclude any interference by this. However, additional IF staining should also be performed to arrive at a definitive conclusion.

4.2 Cytokine stimulation effects on the protein synthesis of type I collagen, β_1 integrin and CD44 in cultured human SF and K4IM cells

Cytokine effects on the expression of cell surface and ECM molecules may be an important factor in the pathogenesis of OA. Therefore, the synthesis of type I collagen, β_1 integrin and CD44 was analyzed to gain further insight into this possibility.

4.2.1 Effects on the synthesis of type I collagen

TNFα caused a decrease in the protein synthesis of type I collagen in both cell populations, which was statistically highly significant in human SF in cell culture. Similar TNFα-triggered effects on the synthesis of type II collagen have been described in chondrocytes (54) and could explain the rapid deterioration of the cartilage's ECM in OA. Since type I collagen mutations have been linked to Ehlers-Danlos syndrome, which among other symptoms is characterized by hyper-mobility of the joints (26) and early onset of OA (55), a decreased collagen synthesis within the inner layer of the synovial membrane triggered by pro-inflammatory cytokines in combination with the increased expression of MMP-1 might also lead to some articular instability, thereby aggravating the OA and hastening its progression. Interactions between SF and particular extracellular matrix components such as collagen are mediated by a variety of integrin receptors. Therefore, cytokine triggered effects on the synthesis of β_1 integrin, which can be associated with different α-integrin chains, was investigated.

4.2.2 Effects on the synthesis of β_1 integrin

Stimulation with IL-10 induced the protein synthesis of β_1 integrin in both cultured human and K4IM SF, while TNFα + IL-10 in combination caused a slight decrease. Additionally, TNFα induced the synthesis of β_1 integrin in K4IM cells on a statistically very significant level. Gene expression and protein synthesis levels of β_1 integrin and the associated integrin-linked kinase (ILK) have been directly linked to cancer cell invasiveness as well as keratinocyte migration in wound repair, with higher levels equaling higher invasiveness and higher migratory activity (56,

57, 58). Since the stimulation with TNFα + IL-10 used in this study simulates the cytokine environment within an advanced stage OA joint and did not lead to an overall increase in the protein synthesis levels of β_1 integrin in either cell population, it may be assumed that this surface protein could only be involved in the initial development of pannus-like tissue when TNFα is not yet partially counteracted by a number of anti-inflammatory cytokines and is not responsible for the invasive properties of SF seen in later stages of OA and RA by itself.
However, the altered synthesis pattern of β_1 integrin could affect the gene expression in SF (32) and thereby play a role in the progression of OA. Also, altered concentrations of β_1 integrin in combination with other ECM adhesion molecules like the hyaluronan receptor CD44 might be enough to promote migratory and/or invasive properties of SF in later stages of OA in addition to physiologic cell matrix interactions.

4.2.3 Effects on the synthesis of CD44

The CD44 receptor could be detected in cultured SF and K4IM cells. TNFα caused a slight increase in detectable HA receptor synthesis in both cell populations. Stimulation with TNFα + IL-10 led to a decrease in the detected relative density levels, which was statistically significant in K4IM cells. The slightly elevated CD44 levels in response to stimulation with TNFα might indicate that SF cause compositional change of the synovia during the initial stages of OA by increased degradation of HA, a process in which CD44 takes part (59). Increased expression levels of the HA receptor have also been linked to processes like cell mobility, migration and metastatic tumor growth (35, 36). It may play a similar role in pannus formation: Elevated levels of CD44 might be in part responsible for the migratory properties of SF, especially in the early stages of OA pannus formation (60) and in collaboration with β_1 integrin and/or other cell adhesion molecules. Since TNFα is counteracted by IL-10 in the later stages of this disease, the reduced synthesis of CD44 might indicate the body's attempt to minimize further damage and stop or slow disease progression.

4.3 Effects of hIL-10 adenoviral transduction on cultured human SF and K4IM cells

In vitro experiments and clinical trials have shown that the overexpression of IL-10 holds a lot of promise for the treatment of RA (51, 52). This could also be relevant for the future management of OA. For this reason, the effects of an *in vitro* adenoviral transduction with the human IL-10 gene on the gene expression of catabolic mediators in cultured human SF and K4IM cells were studied.

4.3.1 Effects on the gene expression of IL-6

TNFα strongly induced the mRNA synthesis of IL-6 in cultured human SF whether transduced with either vector or not. The effect was statistically very significant in non-transduced samples.
In K4IM cells, IL-6 appeared to be induced by the transduction process. The additional stimulation of transduced K4IM cells with TNFα had a blocking effect. However, non-transduced cells increased their gene expression of IL-6 in response to TNFα.
These results in both cell populations are contradictory to current scientific literature describing the high potency of IL-10 in blocking the production of IL-6 *in vitro* (61, 62). However, the literature primarily mentions the use of adenoviral vectors containing vIL-10, which might have a higher therapeutic potency (61, 62), a fact which could have relevance for future *in vivo* applications. Also, the authors only refer to the use of these vectors in RA, and though the disease process appears to be similar in some aspects, SF might react very differently in the pathological setting of OA. Additionally, the immortalization process may have caused K4IM cells to react so differently to the transduction, which should be kept in mind in future overexpression experiments using viral vectors.

4.3.2 Effects on the gene expression and protein synthesis of IL-10

Both cultured human SF and K4IM cells strongly increased their IL-10 gene expression and protein synthesis in response to the adenoviral transduction with hIL-10 gene. Further stimulation with TNFα led to the highest mRNA expression levels in human SF in cell culture, while this pro-inflammatory cytokine seemed to have an impairing effect on the IL-10 gene expression in K4IM cells. However, the absolute IL-10 protein concentration in the stimulation supernatants after exposure to TNFα was the highest observed in both cell populations, showing statistical significance in both cases. None of the other treatments led to a noteworthy increase in IL-10 levels in either cell population.
These results clearly show that the transduction was successful in both cell types. High data distribution spans prevented statistical significance on the gene expression level.
In cultured human SF, this might be partly caused by inter-individual differences. However, that factor does not apply for K4IM cells. Therefore, it could be that the cells in the different experiments were not all equally transduced, despite attempted standardization of cell numbers between samples and usage of 5,000 adenoviral vectors per cell for optimum transduction results.
Since the results of RTD-PCR and ELISA were congruent in cultured human SF, it might be suggested that there are no further regulatory processes between IL-10 gene expression and

protein synthesis in these cells. However, while the hIL-10 ELISA results clearly differ from the RTD-PCR results in K4IM cells, the hIL-10 ELISA results were largely identical to the ones observed in cultured human SF. This suggests that additional regulatory steps might be involved in the control of IL-10 production in K4IM cells. To the author's knowledge, there is no description available in scientific literature for comparison.

4.3.3 Effects on the gene expression of MMP-1 and MMP-3

MMP-1 and MMP-3 were clearly induced in cultured human SF treated with TNFα. However, the mRNA levels of MMP-3 were considerably lower than those of MMP-1. Transduction with either vector led to a decrease in the gene expression of both MMPs after TNFα stimulation in comparison to the non-transduced, TNFα stimulated samples. The hIL-10 vector had the strongest effect.

In K4IM cells, MMP-1 was also increased in response to stimulation with TNFα. However, transduction with the empty vector seemed to induce the gene expression of MMP-1 by itself and additional stimulation with TNFα led to considerable further increase. Transduction with hIL-10 was not able to block the effects of TNFα on MMP-1. MMP-3 expression was completely unaffected in this experiment and mRNA levels always remained comparable to the control.

These observations show that the transduction with either vector, but especially the hIL-10 vector appears to have a down-regulatory effect on the gene expression of MMP-1 and MMP-3 in cultured human SF, which might have applications in the future treatment of OA and might be chondroprotective. Similar effects in chondrocytes have already been described (21). K4IM cells on the other hand reacted entirely differently, which suggests that their response to the transduction has been altered by their immortalization or some other unknown factors.

5. Conclusion & Outlook

All in all, it could be clearly shown that human SF in cell culture and K4IM cells were activated by stimulation with TNFα alone or in combination with IL-10, while the stimulation with IL-10 alone had nearly no effect on the expression of the analyzed genes in either experimental setting. The results obtained were not always in line with those obtained in experiments on cultured human chondrocytes, which might indicate cell specific differences in the gene expression patterns in response to different types of stimulatory cytokines.

The cytokine stimulation also affected the protein synthesis levels of IL-6, IL-10, MMP-1, MMP-3, type I collagen, β_1 integrin and CD44 in both cell populations. IF of the antigens IL-6, IL-10, MMP-1 and MMP-3 in cultured human SF showed results that were largely identical to those obtained in RTD-PCR. However, FC findings in both human SF in cell culture and K4IM cells were somewhat different to the expectations that arose from RTD-PCR. This discrepancy might indicate a procedural error or a problem with the secondary antibodies used in FC.

WB revealed that the protein synthesis levels of type I collagen, β_1 integrin and CD44 in cultured human SF and K4IM cells can be affected by cytokines. These surface proteins might play a role in the pathogenesis of OA with regard to the formation of pannus-like tissue and should be further investigated.

The transduction experiment with hIL-10 was successful and both human SF in cell culture and K4IM cells greatly increased their gene expression and protein synthesis of IL-10. However, this anti-inflammatory cytokine was unable to completely block IL-6, MMP-1 and MMP-3. Therefore, it seems unlikely that overexpression of IL-10 alone would be sufficient for an effective OA treatment. However, the effects of hIL-10 and vIL-10 on macrophages and other leukocytes should also be studied before any definitive conclusion is reached.

K4IM cells appear to be suitable substitutes for cultured human SF in cytokine stimulation experiments. However, they might not be appropriate replacements in transductions and results obtained in this kind of experiment should be interpreted with caution.

Additional investigation is required to deepen the understanding of cytokine interactions and their effect on both the gene expression and protein synthesis in OA. These experiments should include longer variations in the cytokine stimulation periods (e.g. 48 hours, 72 hours etc.), variations of cytokine concentrations, investigation of other catabolic, anti- and pro-inflammatory mediators (e.g. IL-4, VEGF, IL-1β) both as stimulants and effectors and co-cultures containing human chondrocytes to better simulate the intercellular interactions within the joint in their complexity and duration. The FC experiments should be repeated with a

different set of antibodies to exclude procedural error in both cell populations. Finally, K4IM cells should be immunolabeled for IF to determine if they show the same staining patterns as human SF in cell culture.

Moreover, the gene expressions of type I collagen, β_1 integrin and CD44 should be investigated and further cell adhesion molecules (e.g. other subtypes of integrins, cadherins and selectins) should be evaluated for their possible role in OA pathology.

Adenoviral transduction still appears to be an attractive treatment option in the future management of OA, but since hIL-10 on its own was unable to block all the inflammatory responses, other anti-inflammatory mediators (e.g. IL-4, IL-13, soluble TNF-receptor) or their combination as well as vIL-10, which is reported to have greater potency, should be tested for their therapeutic properties. Finally, these experiments could also be performed on SF from healthy donors to see if prolonged exposure to the intra-articular cytokine cocktail in OA has affected the gene expression and protein synthesis patterns in the cultured human SF used in this study.

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7. Declaration on oath

„I, Ingo Mrosewski, declare under oath and by my own signature, that I wrote the submitted dissertation with the title: „Cytokine-mediated expression of catabolic and pro-inflammatory mediators in synovial fibroblasts with regard to the pathogenesis of osteoarthritis“ by myself and without any undisclosed help by third parties and that I did not use any other than the indicated references or resources.

All text passages, that originate verbatim or in sense from publications or presentations of other authors, have been marked with correct citation (see „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*). The passages concerning methods (especially practical work, lab findings, statistics) and results (especially figures, graphics and tables) correspond to URM (see above) and I take responsibility for them.

My contributions to publications originating from this dissertation correspond to those, mentioned in the following joint declaration with my supervisor. All publications originating from this dissertation and of which I am author correspond to URM and I take responsibility for them.

I am aware and apprised of the meaning of this declaration on oath and the criminal consequences of a false declaration (§ 156,161 of the German penal code).”

Date Signature

8. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

9. Complete list of publications

Ingo Mrosewski has contributed in the following way to the following publications:

"Kongressbeitrag" 1:

I. Mrosewski, C. Conrad, N. Jork, E. Wiegand, T. John, W. Ertel, G. Schulze-Tanzil

Regulation of key mediators associated with osteoarthritis by TNFα and IL-10 in primary human synovial fibroblasts

European Students' Conference Berlin, 21.09.2011 – 24.09.2011

Contribution in detail: collection of primary data, writing of the abstract, poster design, oral poster presentation

"Kongressbeitrag" 2:

N. Jork, I. Mrosewski, C. Conrad, E. Wiegand, T. John, W. Ertel, G. Schulze-Tanzil

Regulation von Arthrose-assoziierten Schlüsselmediatoren durch TNFα und IL-10 in primären humanen Synovialfibroblasten

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Contribution in detail: partial collection of primary data, writing of the abstract

Publication (submitted to and accepted by Cell & Tissue Research):

I. Mrosewski, N. Jork, K. Gorte, C. Conrad, E. Wiegand, B. Kohl, W. Ertel, T. John, A. Oberholzer, C. Kaps, G. Schulze-Tanzil

Regulation of OA associated key mediators by TNFα and IL-10: Effects of IL-10 over-expression in human synovial fibroblasts and a synovial cell line

Cell & Tissue Research, March 2014

Contribution in detail: partial collection of primary data, writing of the abstract and the major portion of the paper

Signature, date and stamp of the supervising professor

Signature of the doctoral student

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