Tenocytes, pro-inflammatory cytokines and leukocytes: a relationship?

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Summary

Leukocyte derived pro-inflammatory mediators could be involved in tendon healing and scar formation. Hence, the effect of autologous leukocytes (PBMCs, peripheral blood mononuclear cells and neutrophils) on primary rabbit Achilles tenocytes gene expression was tested in insert assisted co-cultures.
Subsequently, tenocytes gene expression of extracellular matrix (ECM) components (type I collagen, decorin, fibronectin), the cell-ECM receptor β1-integrin, the angiogenic factor myodulin, ECM degrading matrix-metalloproteinase (MMP)1 and pro-inflammatory cytokines (interleukin [IL]-1β, tumour necrosis factor [TNFα] and IL-6) was analysed.
The only significant effect of leukocytes on tenocytes ECM genes expression was a suppression of type I collagen by neutrophils combined with TNFα stimulation. The same effect could be observed analysing the β1-integrin and myodulin gene expression. However, PBMCs up-regulated significantly cytokine and MMP1 gene expression in tenocytes. These *in vitro* results suggest that mononuclear cells could present an exogenic stimulus for the induction of pro-inflammatory and catabolic mediators in tendon.

Key words: cytokines, IL-1β, leukocytes, myodulin, tendon, TNFα

Abbreviations

ABI	applied Biosystems
BSA	bovine serum albumine
DAPI	4',6- diamidino-2-phenylindole
ECM	extracellular matrix
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
HE	haematoxylin Eosin
IFN-γ	interferon-γ
IL	interleukin
MMP	matrix-metalloproteinase
NSAID	non-steroidal anti-inflammatory drugs
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
STAT3	signal transducer and activator of transcription
TBS	tris buffered saline
TGF-β	transforming growth factor-β
RT	room temperature
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Introduction

Tendon rupture and injury leads to a posttraumatic inflammatory response characterized by the presence of multiple pro-inflammatory cytokines such as IL-1β and TNFα as well as cell damage, cell loss and ECM disintegration at the site of tissue injury (1). Additionally blood clot formation, invasion of extrinsic cells and proliferation within the tendon rupture callus, ECM neo-synthesis and angiogenesis can be observed during tendon healing (2) (Fig. 1A). The main ECM component in tendon is type I collagen. The most important proteoglycan in tendon is decorin (3). Fibronectin is an ECM glycoprotein which is expressed by tenocytes involved in their adherence to the ECM (4). Fibronectin and integrin expression is up-regulated during tendon healing (5). The cell-ECM receptor β1-integrin mediate the cell-ECM interaction as an important pre-requisite for cell survival as shown in another connective tissue cell species, the chondrocytes (6, 7). During tendon healing a neoangiogenesis can be observed in the forming granulation tissue (8). Myodulin is a proangiogenic factor, which seems to promote blood vessel proliferation in the region of the myotendinous junction (9). To sustain loading and mediate regular function a remodelling of the rup-

ture callus is necessary (2). Callus remodelling in injured tendons requires more than three months in the rabbit model (8). Remodelling is mediated by ECM degrading enzymes such as MMP1. Particularly MMP1 which is well known to be able to cleave type I collagen has been shown in tendon and cultured tenocytes (10).

The impact of the inflammatory response on the tendon healing outcome is still unclear. TNFα and IL-1β stimulate tenocytes *in vitro* to produce further pro- and anti-inflammatory cytokines e.g. IL-1β, TNFα, IL-6 and IL-10 and ECM degrading enzymes such as MMPs (11,12). This suggests an autoactivation of tenocytes by exogenic cytokines. However, apart from IL-1β and TNFα multiple other cytokines are released by leukocytes. Krause et al., (13) reported recently that e.g. IL-2, IL-8, IL-10, IL-17, TNFα, interferon (IFN)-γ, and granulocyte-macrophage colony stimulating factor (GM-CSF) are produced by PBMCs. Some of them might probably be involved in tendon healing. The cytokine concentration produced by leukocytes might differ from that usually used in *in vitro* experiments with isolated recombinant cytokines. PBMCs consist of monocytes and several lymphocyte subpopulations.

Despite the stimulatory effect of platelets and their extracts on tendon healing is meanwhile intensely discussed (14-16), the effect of exogenic pro-inflammatory mediators such as cytokines released by leukocytes on tenocytes has not yet been studied in tendon tissue and tenocytes at all. Hence, this study was undertaken to assess the direct interactive response of Achilles tenocytes to leukocyte-derived soluble mediators in an indirect co-culture setting.

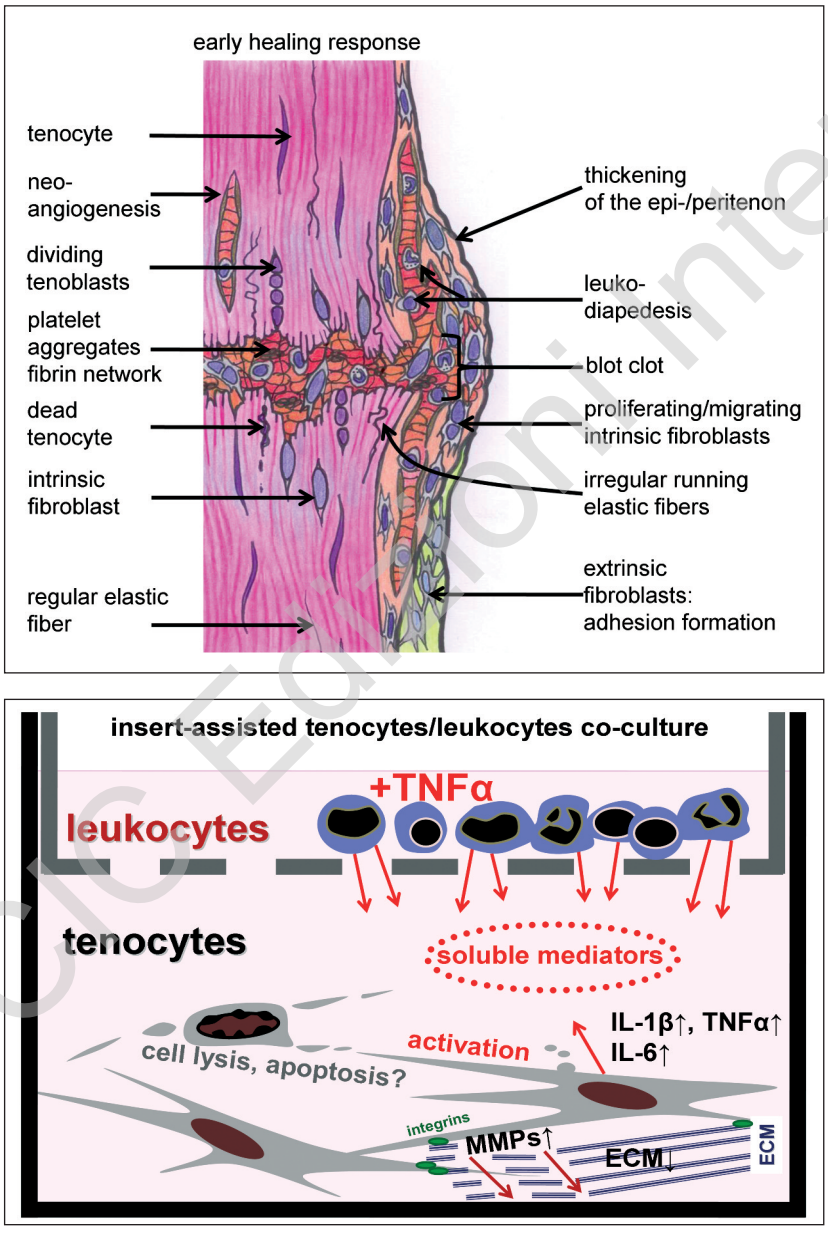

Figure 1A-B. Simplified scheme depicturing aspects of early tendon healing and tenocytes/ leukocytes co-culturing using insert culture.

Materials and Methods

Tenocytes isolation and culture

Rabbit primary Achilles tenocytes were isolated as described previously (17,18) from the midsubstance of seven Achilles tendons of healthy one year-aged adult female donors *post mortem*. This study was approved by the Berlin review board for experiments with animals. The identity of the isolated tenocytes was confirmed by immunolabelling of tenomodulin and tenascin C. Cultures were grown at 37°C in a humidified atmosphere with 5% CO_2 and the growth medium was changed every three days. Growth medium for tenocyte culture consisted of Ham's F-12/Dulbecco's modified Eagle's medium (50/50, Biochrom-Seromed, Munich, Germany) containing 10% fetal calf serum (FCS, Biochrom-Seromed), 25 μg/mL ascorbic acid (Sigma-Aldrich, Munich, Germany), 50 IU/mL streptomycin, 50 IU/mL penicillin, 2.5 μg/mL amphotericin B, essential amino acids, L-glutamine (all: Biochrom-Seromed).

Immunofluorescence labelling of tenocytes

Cover slips seeded with tenocytes were fixed in 4% paraformaldehyde, then rinsed in Tris buffered saline (TBS: 0.05 M Tris, 0.015 M NaCl, pH 7.6) before blocked with protease-free donkey serum (5% diluted in TBS) for 30 min at room temperature (RT), rinsed and incubated with the polyclonal goat tenomodulin (Santa Cruz Biotechnology, Inc., USA),

monoclonal mouse tenascin C antibody BC-24 (GeneTex Inc, Biozol, Eching, Germany), polyclonal rabbit type I collagen antibody (27.5 μg/mL, Acris Antibodies, Herford, Germany) and polyclonal rabbit decorin (0,5 mg/mL, 1:40, Acris, Hiddenhausen, Germany) antibodies in a humidifier chamber overnight at 4°C. Sections were subsequently washed with TBS before incubation with donkey-anti-rabbit-Alexa-Fluor®488 (10 mg/mL, Invitrogen) secondary antibody for 30 min at RT. Negative controls included omitting the primary antibody during the staining procedure. Cell nuclei were counterstained using 4',6- diamidino-2-phenylindole (DAPI) (0.1 μg/mL, Roche). Cover slips were rinsed several times with TBS, embedded with Fluoromount G (Southern Biotech, Diagnostica, Birmingham, USA) and examined using fluorescence microscopy (Axioskop 40, soft imaging solution, Muenster Carl Zeiss, Jena, Germany). Images were taken using a XC30 camera system (Olympus, Germany).

Isolation of peripheral blood mononuclear cells and granulocytes

7 mL heparinized rabbit whole blood was centrifuged for 10 min at 400 g. 3 mL sedimented blood cells were mixed with 12,5 mL solution B (2% FCS in phosphate buffered saline [PBS], 100 U/mL polymixin B). Resuspended blood cells were added to 7.5 mL Biocoll (Biochrom-Seromed) and sedimented for 30 min at 400 g. The interphase was separated and mixed with 12.5 mL solution B. The cell pellet containing the red blood cells and granulocytes (predominately neutrophils) was used for isolation of the neutrophils (see below). The PBMCs were washed in solution B. To isolate the neutrophils, the pellet was mixed with 40 mL solution B. 6.6 mL dextran was added and the suspension left to form sediment for 1 h. The supernatant was then separated and mixed with solution B, before being centrifuged at 600 g for 10 min. Leukocytes were counted and immediately cryo-preserved until use.

HE staining of tendon, tenocytes and leukocytes

Haematoxylin Eosin (HE) staining were performed using paraffin-sections of tendon, cover slips seeded with tenocytes or cytospines of leukocytes as described previously (8).

Two-chamber co-culture of tenocytes and leukocytes

A two-chamber system was used for co-culturing consisting of 6-well plates and inserts (pore size 0.4 μm) (Beckton Dickinson, Franklin Lakes, USA) (Fig. 1B). For the experiments tenocytes were cultured at 15000/cm^2 in 6-well plates for 24 h, subsequently serum-starved (1% FCS containing growth medium) for 2 h. The cryo-preserved autologous leukocytes were gently thawed, pelleted in serum-starved medium and then 4 mio leukocytes, suspended in 1% FCS growth medium per well were added to the insert serving as upper chamber. Some of the co-cultures were stimulated with 10 ng/mL human TNFα (peproTech EC Umkirch, Germany) under similar conditions.

RNA isolation from tenocytes

The insert containing the leukocytes was removed from the 6 well plates. Tenocytes were rinsed with PBS and incubated 5 min with RLT buffer (Qiagen, Hilden, Germany) + 10% mercaptoethanol. Total RNA was isolated using Qiagen RNA isolation mini kit (Qiagen) and the RNA quantity and quality was evaluated using the RNA 6000 Nano assay (Agilent Technologies, Waldbronn, Germany). Reverse transcription was performed using the Quanti Tect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen).

Messenger RNA analysis by real time detection polymerase chain reaction

Real time detection polymerase chain reaction (RTD-PCR) analyses were performed to obtain quantitative gene expression data. To assess the type I collagen gene expression specific primers for the type I collagen and the house-keeping gene β-actin both obtained from the company Qiagen were used. Specific primers for myodulin, decorin, fibronectin, β1-integrin, IL-1β, TNFα, IL-6, MMP1 and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (3) gene sequences (Tab. 1) in the rabbit were used which derived from Applied Biosystems (ABI), Foster City USA. Type I collagen gene expression was normalized *versus* β-actin, expression of the other genes *versus* GAPDH. For RTD PCR, 1 μL aliquots of the cDNA were amplified using RTD-PCR in a 20 μL reaction mixture using the Quantitec Probe RTD-PCR kit (Qiagen) or TaqMan Gene Expression Assay. All assays were performed in an Opticon 1 – Real-Time-Cycler (Opticon™ RTD-PCR, Biorad). The conditions for TaqMan analyses were: 10 min at 90°C, and then for 41 cycles 15 s at 95°C, 30 s at 56°C and followed by 6°C cooling. The lack of primer dimers and the specifity of amplification were further confirmed by efficacy testing using rabbit Achilles tendon cDNA and agarose gel electrophoresis of PCR products all showing a single band of expected size. Relative gene expression levels were normalized *versus* the housekeeping genes and calculated with the $2^{-deltaCT}$ method (19).

Table I. Oligonucleotides used for RTD PCR analysis with rabbit tenocytes cDNA.

		forward/reverse primer	probe/reference	bp
β-actin	Qia	TGGGACGACATGGAGAA GAAGGTCTCAAACATGATCTGG	GCCCCCCTGAACCCTAA	147
type I collagen	Qia	GGCAACGATGGTGCTAA GACCAGCATCACCTCTGTC	AATGCCTGGTGAACGTG	138
GAPDH	ABI	NA	Oc03823402_g1	82
decorin	ABI	NA	Hs00370384_m1	77
fibronectin	ABI	NA	Hs01549940_m1	66
myodulin	ABI	NA	Oc03399505_m1	146
β1-integrin	ABI	NA	Hs00559595_m1	75
TNFα	ABI	NA	Oc03397715_m1	74
IL-1β	ABI	NA	Oc03823250_s1	86
IL-6	ABI	NA	Oc04097053_m1	72
MMP1	ABI	NA	Oc03398637_m1	64

ABI: Applied Biosystems, Qia: Qiagen, NA: not available

Western blot analysis

Western blotting was used to determine tenocytes β1-integrin and MMP1 protein synthesis when co-cultured with leukocytes for 24 h, stimulated with TNFα or remained untreated as a control. Tenocyte monolayers were washed with PBS solution, whole cell proteins were extracted by incubation with lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 5 mM $CaCl_2$, 2 mM DTT, 1 mM EGTA [all: Carl-Roth, Karlsruhe, Germany] and proteinase inhibitors [proteinase complete mini, Roche]) on ice for 30 min. Cell debris was removed by centrifugation. Supernatants were stored at –80°C until use. Total protein concentration of whole cell extracts was normalized using Bradford protein assay (Roti-Nanquant, Carl-Roth) and bovine serum albumin (BSA) as a standard. Samples were separated by Tris-glycine SDS-PAGE (12% acrylamide) under reducing conditions before being transferred to a nitrocellulose membrane (Carl-Roth), using a transblot apparatus (Bio-Rad). Equal protein loading was controlled by the use of Ponceau S staining (Sigma-Aldrich) and β-actin house-keeping protein expression. Membranes were blocked using blocking buffer (3% BSA/ PBS/ 0.05% Tween20) for 1 h at RT and incubated overnight at 4°C with primary antibodies (mouse anti-β1-integrin [Millipore Corporation/Chemicon International, Billerica, USA] monoclonal mouse anti-MMP1 [25 μg/ml, R&D systems, USA] or monoclonal mouse anti-β-actin [Sigma-Aldrich]) diluted 1:1000 in blocking buffer. Membranes were washed with PBS/ 0.05% Tween20 and incubated with horseradish peroxidase conjugated with secondary goat-anti-mouse IgG antibodies (1:5000, Dako, Glostrup, Denmark) for 2 h at RT. Specific binding was detected by chemiluminescence using RotiLumin™ (Carl-Roth) and X-ray films. The developed films were scanned and protein bands were semi quantified by densitometric scanning (AlphaDigiDoc, Cell Biosciences).

Statistical analysis

Normalized data were expressed as the mean and error of mean (mean ± SEM). Differences between experimental groups were considered significant at $p<0.05$ as determined by student`s paired two-tailed t test (GraphPad Prism 5, GraphPad software inc, San Diego, California, USA).

Results

Characterization of rabbit Achilles tendon and tenocytes in vitro

Rabbit Achilles tenocytes are lying in rows between bundles of ECM fibers and possess elongated mostly heterochromatic cell nuclei (Fig. 2A). The tenocytes in tendon were smaller than isolated and monolayer cultured primary rabbit Achilles tenocytes (Fig. 2B). Cultured tenocytes exhibited an elongated shape, rounded mostly euchromatic cell nuclei and possess long and sheet-like cytoplasmic cell appendages by which they communicated with their neighbouring cells (Fig. 2B, 3A). Isolated rabbit leukocytes were much smaller than tenocytes (Fig. 2A-D). Rounded or kidney-like nuclei with small cytoplasmic margins were discernible in PBMC preparations. Typical multi-segmented nuclei could be observed in granulocytes cell preparations. Some eosinophils were also detectable in the granulocytes preparations (Fig. 2C-D). Rabbit Achilles tenocytes expressed at the protein level the anti-angiogenic tendon marker tenomodulin (20), the ECM glycoprotein tenascin C, the main ECM protein type I collagen, the ubiquitous protein fibronectin (not shown) and the typical tendon proteoglycan decorin (Fig. 3A-D).

ECM gene expression in tenocytes/leukocytes co-culture

Irrespective of the treatment course, the rabbit tenocytes expressed myodulin, which is known to be expressed at the myotendinous junction and to mediate angiogenesis (9). When tenocytes were stimulated with neutrophils combined with TNFα they down-regulated their type I collagen, myodulin and β1-integrin gene expression significantly (Fig. 4A,D, 5A). At a non significant level co-culturing with PBMCs impaired tenocytes decorin gene expression and the TNFα treatment induced slightly decorin and fibronectin expression (Fig. 4B,C). Effects on β1-integrin protein expression were not significant (Fig. 5B).

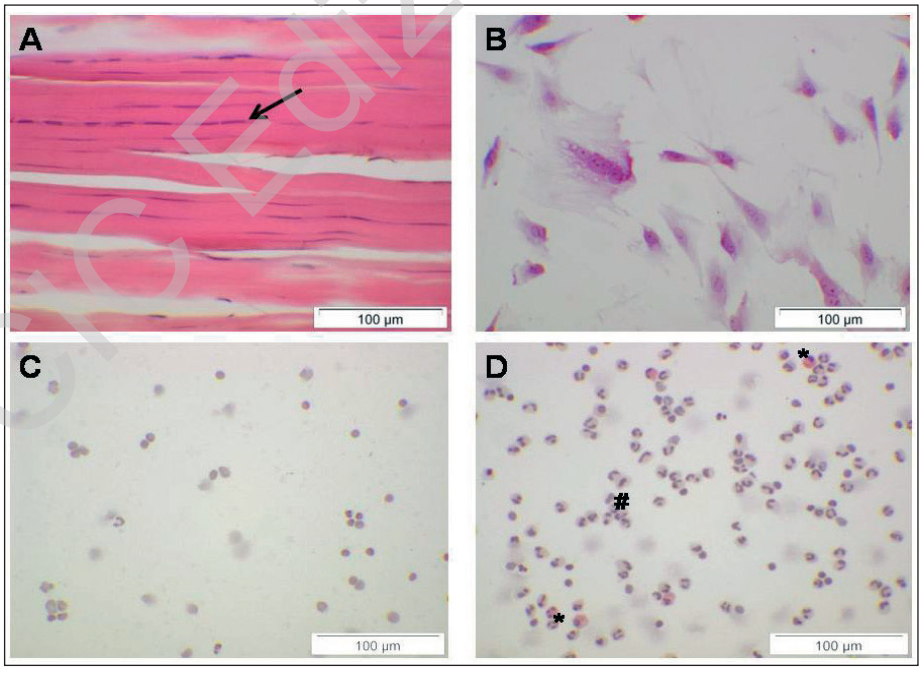

Figure 2A-D. Tenocytes and leukocytes.

Cytokine gene expression in tenocytes in response to autologous leukocytes co-culture

PBMCs up-regulated significantly tenocytes gene expression of the key pro-inflammatory cytokines investigated here: IL-1β, TNFα, and IL-6 (Fig. 6A-C). The combination of PBMCs with TNFα had a further inductive, but not any longer significant effect. TNFα stimulation had an inductive effect on rabbit tenocytes cytokine expression which did not achieve significance. When the tenocytes were co-cultured with neutrophils without TNFα stimulation, tenocytes cytokine expression was induced but the effect was not significant. The combination of neutrophils with TNFα in co-culture with tenocytes lead to lower mean expression values of cytokines compared with the treatment with neutrophils alone.

MMP1 expression in tenocytes/leukocytes co-culture

The ECM degrading enzyme MMP1 was significantly up-regulated in the tenocytes/PBMCs co-cultures (Fig. 7A). The stimulation with TNFα or neutrophils induced also MMP1 expression in tenocytes but to a lesser (non significant) extent. The protein expression of MMP1 shown by western blotting revealed mostly similar results despite not significant (Fig. 7B).

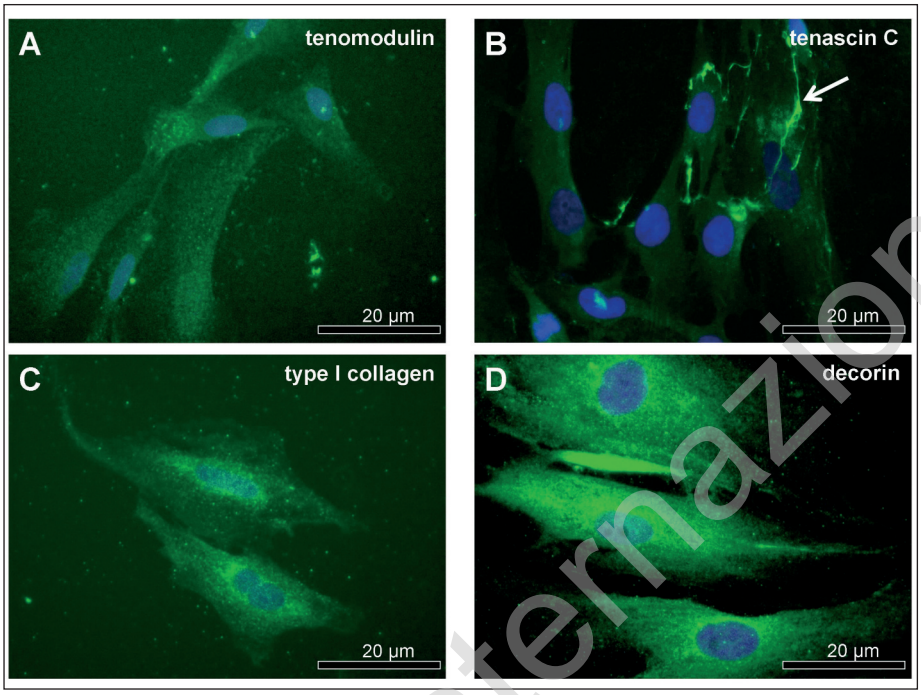

Figure 3A-D. Characterization of cultured tenocytes.

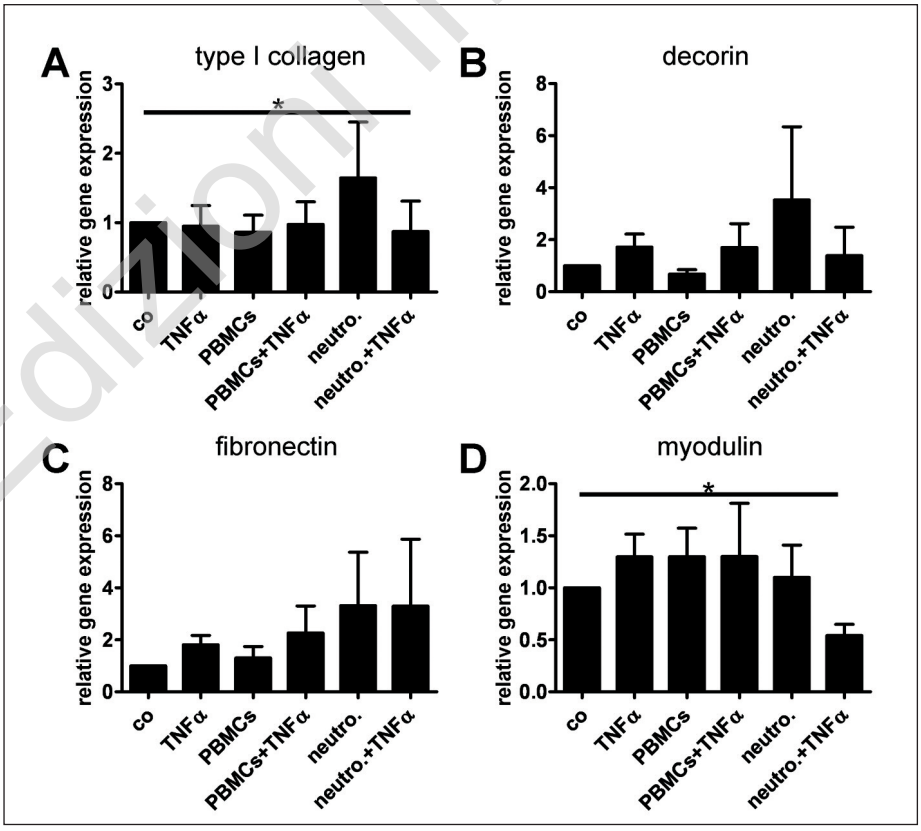

Figure 4A-D. Type I collagen, decorin, fibronectin and myodulin gene expression in tenocytes co-cultured with leukocytes.

Discussion

Healing of ruptured tendons is influenced by the early post-traumatic inflammatory response (Fig. 1A), whereby the pro-inflammatory cytokines IL-1β, TNFα and other immunoregulatory cytokines such as IL-6 might play a role. Generally, tendon healing is a time consuming reparative process leading to scar tissue formation whereby scars are associated with

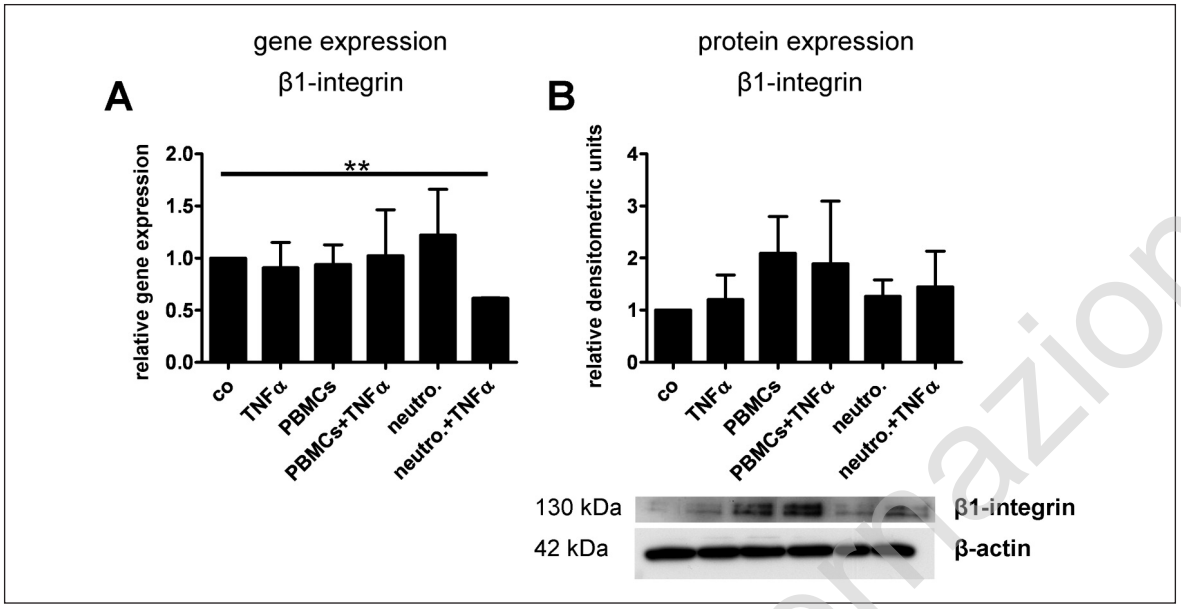

Figure 5A-B. β1-integrin gene and protein expression in tenocytes co-cultured with leukocytes.

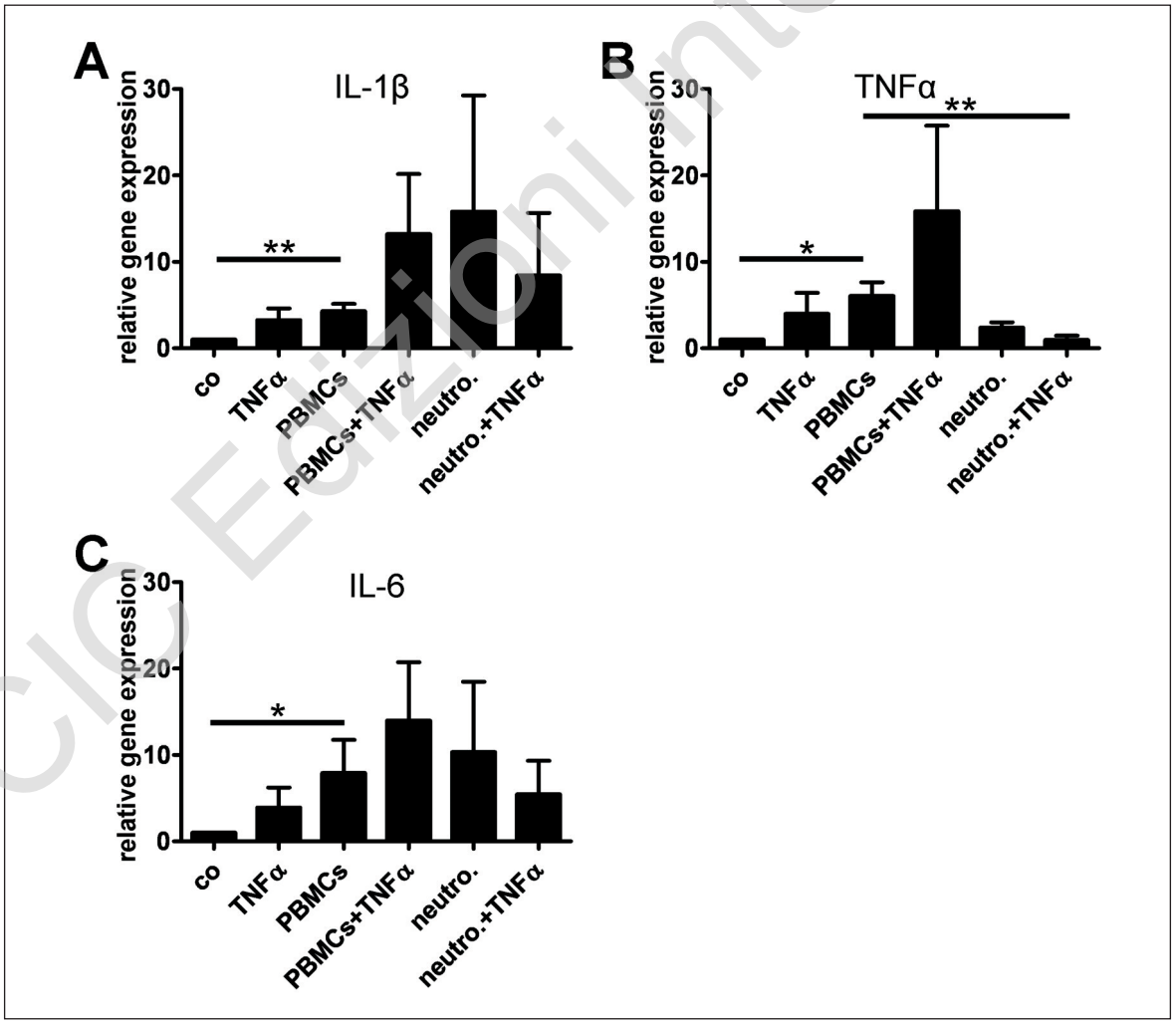

Figure 6A-C. Cytokine gene expression in tenocytes co-cultured with leukocytes.

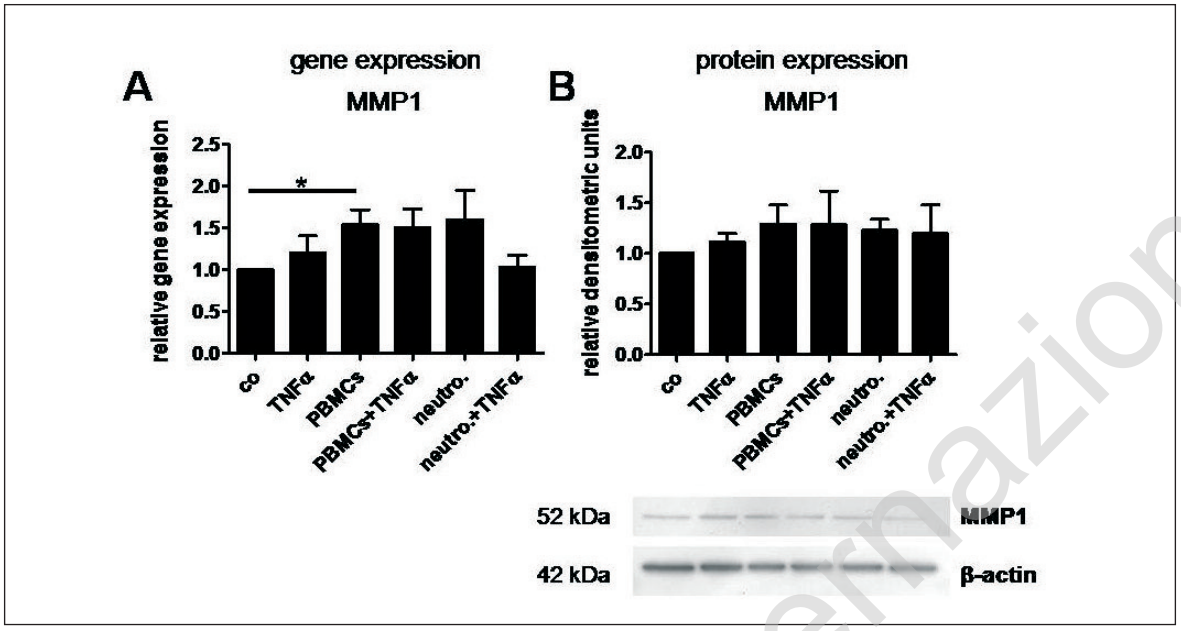

Figure 7A-B. MMP1 gene and protein expression in tenocytes co-cultured with leukocytes.

an altered function of tendon. IL-1β and TNFα are able to strongly activate tenocytes to up-regulate their gene expression for multiple cytokines such as IL-1β, TNFα, IL-6 and IL-10 via auto- and paracrinic amplification loops. Additionally, these cytokines induce ECM degrading enzymes such as MMPs and suppress their type I collagen synthesis initiating a catabolic cascade (11,12).

Immigrating leukocytes are an important exogenic source for pro-inflammatory cytokines in healing tendons. They are directly released during post-traumatic bleeding or recruited from blood vessels by chemotactic signals due to tissue damage (Fig. 1A). Hence, in this study rabbit tenocytes were co-cultured with autologous leukocytes to study the effect of leukocytes on tenocytes induced by soluble mediators. The rabbit was used as a cell source since it is a common experimental surgery model to study tendon repair. Additionally, rabbit tenocytes are also well characterized *in vitro* (21). Tenocytes were combined with autologous leukocytes in the present study to exclude effects of an immunogenic response against allogenic cells influencing the results of co-culturing. However, when as a confirmation experiment human hamstring tenocytes were combined with allogenic human blood-derived leukocytes similar effects in regard to tenocytes cytokine induction by PBMCs were detectable (not shown). PBMCs induced the gene expression of the ECM degradative enzyme MMP1, the pro-inflammatory cytokines IL-1β, TNFα and IL-6 in tenocytes. MMP1, once activated, can cleave type I collagen. Hence, it is important for callus remodelling in the advanced tendon healing phase. However, the activation of MMP1 was not assessed in the present study: the non-glycosylated 52 kDa pro-enzyme was detected by western blotting. IL-1β is a typical pro-inflammatory cytokine, up-regulated in injured tendons (22). A blockade of TNFα improved early tendon healing in a rotator cuff healing model in the rat (23). In contrast, IL-6 has been implicated to play essential roles in the wound healing process (24). Tendon healing was reduced in IL-6 knock out mice (25). Nakama et al. (26) showed that IL-6 promotes blood vessel proliferation induced by vascular endothelial growth factor (VEGF)-dependent angiogenesis via the Signal Transducer and Activator of Transcription (STAT3) signalling pathway. STAT3 activation may therefore play a critical role in tendon adaptation to ischaemic stress by maintaining the intratendinous vasculature after tendon injury. Since IL-6 can increase vascular permeability, leading to oedema, this may explain the occurrence of swelling which is a typically feature in the first hours and days after a tendon injury and maybe necessary for the wound healing processes.

Myodulin is a pro-angiogenic factor and could be demonstrated in the present study in cultured rabbit tenocytes. Myodulin expression was not restricted to the myotendinous junction as suggested by (9) since in the present study the midsubstance of tendon was used for tenocyte isolation. However, tenocytes myodulin gene expression was neither induced by PBMCs nor by neutrophils in a significant manner but rather suppressed by neutrophils combined with TNFα.

Peripheral blood-derived leukocytes were used in the present study, but one can assess that leukocytes immigrating in response to chemotactic signals and hence, activated, might present a different cytokine expression profile and release further growth factors and mediators influencing tendon healing. Leukocytes cytokine expression pattern might also be influenced by direct cell-cell contacts between tenocytes and leukocytes and hereby, lead to a modified/enhanced response of tenocytes. So a direct co-culture system should be applied in future studies. Additionally, the soluble mediators released by the autologous leukocytes into the culture supernatants should be further characterised to identify key effector cytokines and chemokines. Not only typical pro-inflammatory cytokines but also growth factors are released by leukocytes such as transforming growth factor (TGF)β1 or vascular endothelial growth factor (VEGF) (14,27). In comparison to the effect of leukocytes, teno-

cytes were stimulated with TNFα alone. At all the effect by TNFα alone was weaker compared with that of PBMCs. Since human TNFα was used a reduced stimulatory effect on rabbit tenocytes can be supposed caused by interspecies differences in susceptibility. However, a mixture of several soluble mediators released by the PBMCs might exhibit additive effects when tenocytes were exposed to them. Irrespective of the presence of growth factors produced by leukocytes, tenocytes ECM and β1-integrin gene expression revealed no major changes except for a suppression of type I collagen and β1-integrins by neutrophils combined with TNFα. However, the inconsistency between β1-integrin gene and protein expression level might depend on translational and posttranslational regulatory effects.

The, probably, divergent effects of particular PBMC effector cell subpopulations should be further analysed in future. The neutrophil cell fraction contained also some eosinophil granulocytes. This heterogeneicity might be responsible for the high standard deviations observed in response to stimulation of tenocytes with neutrophils.

Conclusion with some perspectives for the future

Taken together, this study indicates an interplay between leukocytes and tenocytes via soluble mediators: tenocytes can be activated by leukocytes to express several pro-inflammatory cytokines, MMPs and probably other factors. The particular impact of this interplay on tendon healing should be further investigated. Nevertheless, it should be considered that the behavior of peripheral blood derived leukocytes may be quite different if they are stimulated/ activated by the *in vivo* injury environment. Similarly, the tenocytes may respond differently in an injured tendon in contrast to the *in vitro* conditions studied here. When comparing the setting with *in vivo* conditions, tenocytes stress and injury might amplify the susceptibility of them to leukocyte derived mediators. Furthermore, the consequence of direct cell-cell contacts between both cell types remained up to now disregarded in this study.

The implications of the expression of pro-inflammatory mediators by tenocytes should be addressed in ongoing experiments to answer the question whether these factors ultimately impair the tendon reparative process or whether they are required for an effective tendon callus remodeling. The use of non-steroidal anti-inflammatory drugs (NSAID) is a common clinical practice (28). Hence, the possible effects of anti-inflammatory medications, e.g. the application of particular NSAIDs following tendon injury could be addressed in this model in future. It has been shown that both, the prostaglandin (PG) E2 release, but also the adaptive collagen synthesis in tendon was inhibited by NSAIDs during exercise (29). In addition, adhesion formation was reduced in healing tendons by the NSAID ibuprofen. On the contrary, tenocyte proliferation and migration was impaired and MMP expression was induced in cultured tenocytes whereby the expressions of types I and III collagen remained mostly unaffected (28,30-32). The results of these previous studies suggest that anti-inflammatory therapy with NSAIDs might also interfere with some features of the natural healing response in tendon.

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