Original Article

Marked expression of TNF receptors in human peritendinous tissues including in nerve fascicles with axonal damage - Studies on tendinopathy and tennis elbow

C. Spang[1,2], L. Renström[1], H. Alfredson[3,4,5], S. Forsgren[1]

[1]Department of Integrative Medical Biology, Anatomy Section, Umeå University, Umeå, Sweden; [2]Dr. Alfen Orthopaedic Spine Center, Würzburg, Germany; [3]Department of Community Medicine and Rehabilitation, Sports Medicine Unit, Umeå University, Umeå, Sweden; [4]Pure Sports Medicine Clinic, London, UK; [5]ISEH, UCLH, London, UK

Abstract

Background: The peritendinous connective tissues can have importance in chronic tendon pain. Recently cytokine TNF-α has been suggested to be involved in tendinopathic processes. It is not known how TNF-α and its receptors TNFR1 and TNFR2 are expressed in peritendinous tissues. **Methods:** The objective for this study was to immunohistochemically evaluate the expression patterns of these in the peritendinous tissue located between the plantaris and Achilles tendons and the one located superficially to the extensor origin at the elbow region for patients with tendinopathy/tennis elbow. **Results:** The nerve fascicles were of two types, one type being homogenously stained for the nerve markers βIII-tubulin and neurofilament and the other showing deficits for these suggesting features of axonal damage. Much more distinct TNFR1/TNFR2 immunoreactions were seen for the latter nerve fascicles. TNFR1 was seen in axons, TNFR2 mainly in Schwann cells. TNFR1 and particularly TNFR2 were seen in walls of parts of blood vessels. The dispersed cells showed frequently TNFR1 and TNFR2 immunoreactivity. **Discussion:** These findings suggest that TNF-α can be related to degenerative events but also attempts for healing concerning the nerve structures. The marked expression of the TNF-α system in the peritendinous tissue suggests an impact of TNF-α in tendinopathy/tennis elbow.

Keywords: TNF, Peritendinous, Tendinopathy, Tennis Elbow, Nerve Degeneration

Introduction

Painful conditions related to the insertions and origins of muscles are frequently occurring. Concerning the condition termed tendinopathy, it has become more and more apparent that the peritendinous tissue, i.e. the connective tissue located adjacent to the tendon tissue proper, is of importance in order to explain the pain symptoms of the tendinopathy. It is thus found that tendon injury related to increased mechanical loading leads to local inflammation and increase in blood flow in the peritendinous tissue[1]. There is actually a very limited

tissue turnover in the tendon core[2]. In studies on the Achilles tendon and the peritendinous tissue located ventrally to this, it was found that the great majority of nerve fascicles of the region were located in the peritendinous tissue, here frequently lying close to blood vessels[3]. Treatments, such as surgical scraping[4], as well as polidocanol injections[5], affecting the peritendinous tissue, have had a positive outcome on the pain in Achilles tendinopathy. In recent studies, it has been shown that the peritendinous tissue located between the Achilles and plantaris tendons in cases where the two tendons are closely apposed in tendinopathy conditions is richly innervated[6]. Frequent blood vessels and numerous dispersed cells were also found in this tissue[7].

It is of relevance to know the factors that are related to the functions of the nerves, the blood vessels and the dispersed cells in the peritendinous tissue in conditions with tendinopathy. In preliminary studies on a small material of peritendinous tissue we noted that the TNF-α system might be of great importance, there being marked expressions of tumor necrosis factor alpha receptor 2 (TNFR2) in blood vessel walls and dispersed cells. Concerning both the tumor

necrosis factor alpha receptor 1 (TNFR1) and TNFR2 we have now extended these studies, hereby focusing on the TNF-α system in relation to the innervation, the blood vessels and the cell populations occurring in the region.

The TNF-α system can have an effect on the nerves, blood vessels and the cells in the tissue in several regions of the body. Concerning innervation, expressions of TNF-α and its receptors have been previously noted for nerves, e.g. those of lung tissue[8]. There are several lines of evidence which suggest that TNF-α can be produced by neurons from an early stage up to fully differentiated stages[9]. This is supported by the occurrence of an anterograde transport of TNF-α from rat DRG to spinal cord and injured sciatic nerve[10,11]. Of relevance is also the known fact that TNF-α can be a mediator of pain[12]. TNF-α neutralization leads to a pronounced antinociceptive effect, the effect being on pain fibers, as seen in studies on a rat model of arthritis[13]. TNF-α can nevertheless have a dual role for the nervous system, being a promoter of neurodegeneration in the striatum but a protector against neurogeneration in the hippocampus[14]. TNF-α can on the whole participate both in nerve degeneration and nerve regeneration[15].

With respect to blood vessels and cells dispersed in a tissue it is well-known that TNF-α is synthesized and released by a large number of cell types such as macrophages, mast cells, fibroblasts and endothelial cells[16]. It is also known that TNF-α can be a mediator that drives blood vessel remodeling in inflammation[17]. The TNF-α system can be upregulated in response to tissue remodeling, one example being the finding of an enhanced immunohistochemical expression of TNF-α and its receptors in the walls of cerebral arteries in rats following experiments with subarachnoid hemorrhage and middle cerebral artery occlusion[18]. TNF-α has also been suggested to stimulate angiogenesis following ischemia[19].

There is no information at all as to whether there are expressions of TNF-α and its receptors TNFR1 and TNFR2 in the peripheral nerves of the peritendinous tissue. There is also no information in the literature on the expressions of these for the blood vessels and dispersed cells in the peritendinous tissue. What is known for tendon core, i.e. tendon tissue proper, is that there is expression of TNF-α, as well as TNFR1 and TNFR2, in the tendon cells[20].

We have in the present study examined the expression patterns of TNF-α, TNFR1 and TNFR2 within the nerve fascicles, blood vessel walls and the dispersed cells in the peritendinous tissue. Two types of peritendinous tissue were examined; the one located between the Achilles and plantaris tendons and the one located at the origin of the common extensors/extensor carpi radialis brevis (ECRB) (tennis elbow region). The hypothesis was that the TNF-α system was expressed in the innervation, as well as for the blood vessels and dispered cells, suggesting that the system is involved in the remodeling processes that occur in the peritendinous tissue.

Table. Pain and function scores from the majority of patients.

	VAS *	VISA-A **
Mean	66,8	45,9
Median	75	46
Range	18-97	23-66
* from 21 patients, ** from 19 patients.		

Methods

Patients

Tissue samples were taken from patients suffering from plantaris-associated Achilles tendinopathy or from pain in the ECRB/common extensor origin at the lateral epicondyle (tennis elbow). Pain symptoms for these patients had lasted for at least 3 months when the biopsies were taken.

Patients with plantaris-associated midportion Achilles tendinopathy showed clinically a tender thickening of the Achilles tendon midportion and Ultrasound and Colour Doppler (US/CD) examination verified midportion Achilles tendinopathy with plantaris tendon involvement[21,22]. In total, 34 plantaris tissue specimens including the peritendinous connective tissue in between the Achilles and plantaris tendons from 30 patients (7 women, 23 men; mean age 47 years; 4 patients with bilateral symptoms) were collected.

The diagnosis tennis elbow was made when there was pain on palpation of the extensor origin, pain elicited from the region on resisted wrist extension and 3rd finger test. Furthermore, US/CD examination showed structural changes and high blood flow inside and outside the extensor origin[23]. Tissue specimens from the fibrous and fat tissue covering the surface of the origin from 4 patients (2 women, 2 men; mean age: 46 years) were taken.

All clinical examinations were carried out by the same experienced doctor (HA).

VAS and VISA-A measurements

For evaluation of the degree of tendinopathy/tennis elbow we initially used the VAS score, evaluating the amount of pain the patient had during loading activity, where 0 was no pain and 100 worst pain. As illustration, a footballer evaluated his/her pain during football activity, and a walker during walking etc. During the last years the more functional VISA-A score was measured. For the VISA-A a low score indicates poor function and a high score indicates high tendon function. In total, 21 patients were measured concerning VAS score and 19 concerning VISA-A score (Table).

Surgical procedures

Surgery was performed by one of the authors (HA). For patients with plantaris-associated midportion Achilles tendinopathy the procedure was as follows: Via a short longitudinal skin incision at the medial side of the Achilles

tendon midportion, the medial side of the Achilles and plantaris tendons was visualised. The plantaris tendon was very closely attached to the Achilles tendon. Due to previous knowledge on existence and importance of adherences and interactions between the two tendons[24-26] the plantaris tendon was removed together with the ventro-medial fatty richly vascularised peritendinous connective tissue located inbetween the plantaris and Achilles tendons ("plantaris speciments"). For more details, see [21,24].

For patients with diagnosed tennis elbow, skin markers were placed to localise the region with high blood flow outside the extensor origin. During the minimally invasive procedure under local anesthesia (3-4 ml of xylocain+adrenaline) the connective tissue from the region with thickened fibrous tissue and high blood flow was released and removed from the surface of the extensor origin.

The removed connective tissue (the connective tissue between plantaris and Achilles tendons and the connective tissue from tennis elbow region as described above) represents the content of the biopsies that were further processed. For matter of simplicity the tissue will for both locations further on be referred to as "peritendinous tissue".

Ethics

This study was approved by the Regional Ethical Board in Umeå (dnr 04-157M; 2011-83-32M). The experiments were performed according to the principles expressed in the Declaration of Helsinki. All patients included gave an informed consent.

Sampling, fixation and sectioning

Straight after surgery the tissue specimens were kept in fixative solution (4% formaldehyde in 0.1 M phosphate buffer, pH 7.0) at 4°C overnight. After that the samples were washed three times in Tyrode's solution containing 10% sucrose (pH 7.2). The first washing step was performed at 4°C overnight.

Before freezing, the samples that were large were divided into smaller pieces. Then they were placed on a thin cardboard surrounded by OCT embedding medium (TissueTek, Miles Laboratories, Naperville, IL, USA). Finally the cardboard with the specimen was put in liquid propane chilled with liquid nitrogen and then stored at -80°C until use.

For immunohistochemical analyses, the specimens were cryosectioned with a thickness of 7 μm (Leica Microsystem CM 300, Heidelberg, Germany) and mounted on superfrost plus slides (Thermo Scientific, Braunschweig, Germany).

Immunohistochemistry

Antibodies and control stainings

Antibodies used were directed to components of the TNF-α system (TNF-α, TNFR1, TNFR2), markers for white blood cells (CD68, neutrophils/T-cells marker, eosinophil peroxidase, mast cells) and nerve markers (βIII-tubulin, neurofilament, S-100β) and marker for fibroblasts.

The TNF-α antibody used was a goat polyclonal IgG raised against a N-terminal peptide mapping of human TNF-α (code sc-1350, dilution 1:50, Santa Cruz Biotechnologies). The TNFR1 antibody was a goat polyclonal IgG raised against the C-terminus peptide mapping of mouse TNFR1 (code sc-1070, 1:100, Santa Cruz). The TNFR2 antibody was a goat polycloncal IgG antibody raised against the peptide mapping at the C-terminus of mouse TNFR2 (code sc-1074, 1:100, Santa Cruz). All these antibodies have been characterized and tested in previous studies on human tendon tissue[20].

The antibodies against macrophages (CD68, code M0814, 1:50, DakoCytomation), neutrophils and T-cells (code MCA805G, 1:100, AbD Serotec), eosinophil peroxidase (code MAB1087, 1:100, Chemicon), mast cells (AA1, code ab2378, 1:100, abcam) and fibroblasts (code M0877, 1:100, DakoCytomation) were mouse monoclonal antibodies. βIII-tubulin, an axonal marker, was detected by a mouse monoclonal antibody (code T8660, 1:300, Sigma-Aldrich). Schwann cells were identified by a mouse monoclonal antibody against S-100β (code S2657, 1:100, Sigma Aldrich). All these antibodies have been successfully tested and used in previous studies[6,7,27-32]. For all antibodies control stainings replacing the primary antibody with PBS were performed.

Staining procedure

All specimens were processed for demonstration of the elements in the TNF-α system (TNF-α, TNFR1, TNFR2). The frozen sections were initially let thawed for about 5 min and were then pre-incubated in potassium permanganate for 2 min to increase visualisation of specific immunofluorescence reaction sites[33]. The slides were then washed three times in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.1% sodium azide as preservative. After that the slides were kept in 1% Triton X-100 in 0.01 M PBS (pH7.4) for 20 min followed by another washing step. Then 5% normal serum was added for 15 min before incubation with the primary antibody at 37°C for 60 min proceeded. After that sections were washed again and normal serum was added followed by incubation with the secondary antibody (37°C, 30 min). Eventually, the samples were washed and mounted with Vectashield mounting medium (H-1000).

Staining for monoclonals was as previously described[31,32]. Rabbit normal serum (code X0902, 1:20, DakoCytomation) and TRITC-conjugated rabbit anti-mouse (code R0276, 1:40, DakoCytomation) was applied. All dilutions were made in 0.1% bovine serum albumin (BSA) in 0.01 M PBS (pH 7.4). For labelling with goat antibodies, donkey normal serum (code 017-000-121, 1:20, Jackson Immune Research Laboratories Inc.) and FITC-conjugated donkey anti-goat secondary antibody (code 705-095-147, 1:20, Jackson Immune Research Inc.) were used. Dilutions were made without BSA.

Parallel sections to those that were processed for immunohistochemistry were stained for demonstration of tissue morphology (hematoxylin-eosin [htx-eosin]).

The microscopical evaluation was carried out using a Zeiss Axioscope 2 plus microscope equipped with epifluorescent
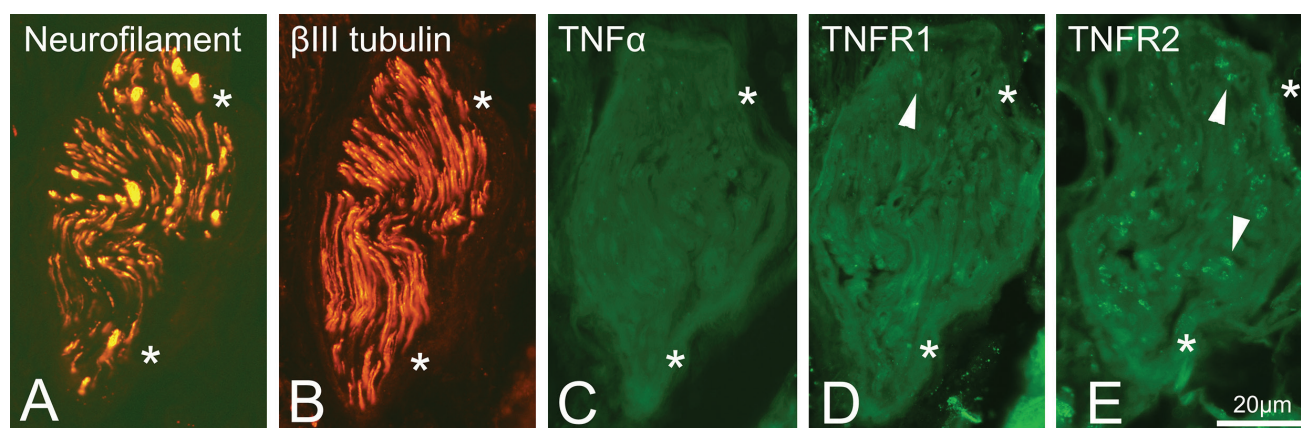
Figure 1. Serially sectioned nerve fascicle in specimen from tennis elbow patient. The nerve fascicle is homogenously stained for neurofilament (A) and βIII tubulin (B). The sections illustrating C-E were processed for TNF-α, TNFR1 and TNFR2 respectively. There is no specific immunoreactivity in (C). There are weak signs of immunoreactivity in (D) (arrowhead). In (E), there are certain immunoreactions visible (arrowheads). Asterisks at corresponding sites of perineurium. There are some autofluorescence spots in (C-E).

technique and an Olympus DP70 digital camera. Figure montages were created by using Adobe Photoshop CS5.

Double staining

Double stainings were performed on a subgroup of chosen sections. The localisation of TNF-α, TNFR1 and TNFR2 within the tissue section was hereby tested via double staining with markers for white blood cells (CD68, neutrophils/T-cells marker, eosinophil peroxidase, mast cell tryptase), nerve fiber marker (βIII-tubulin) and the Schwann cell marker S-100β. The procedures for the double stainings for TNF-α, TNFR1 and TNFR2 and the various mouse monoclonal antibodies were as described previously concerning double stainings with polyclonal antibodies (in this case goat antibodies) and mouse monoclonal antibodies[7,31,32]. When βIII-tubulin and S-100β was targeted no preincubation with potassium permanganate was performed.

Results

Nerve fascicles

Nerve fascicles were observed in the peritendinous tissue of both plantaris and tennis elbow specimens. They were more frequent and usually of larger dimensions in the latter. Fine nerve fibers and perivascular innervation was also observed.

The occurrence or not of distinct nerve fascicles in the sections was related to the magnitude of peritendinous tissue present in the specimens. They were clearly observable in specimens of 11 of the patients. These specimens contained a lot of peritendinous tissue.

The nerve structures were clearly seen in stainings for neurofilament (Figures 1A, 2A) but could also be identified in parallel sections processed for morphology (htx-eosin)

(data not shown). The nerve fascicles were also visualized in stainings for βIII-tubulin (Figures 1B, 2B). Reactions for the elements of the TNF-α system (TNF-α, TNFR1, TNFR2) were detected to different extents in the nerve fascicles. In order to give a clear pattern of these reactions, evaluations were made in relation to those seen in parallel stainings for βIII-tubulin/neurofilament.

It was observed that the nerve fascicles were not always homogenously outlined but that a partial lack of reactions for βIII-tubulin/neurofilament was seen for some. Such nerve fascicles occurred in specimens that also contained homogenously outlined nerve fascicles.

The nerve fascicles that were homogenously outlined in sections processed for βIII-tubulin/neurofilament (Figures 1A, 1B), did not exhibit TNF-α immunoreaction (Figure 1C). Only very limited or sometimes no immunoreactions for TNFR1 (Figure 1D) were displayed in these nerve fascicles. TNFR2 immunoreactions were seen to some extent (Figure 1E) or were very rarely displayed in these. For the nerve fascicles that exhibited a non-uniform βIII-tubulin/neurofilament immunoreaction (Figures 2A, 2B), i.e. parts of the nerve fascicles being non-reactive, clearly more pronounced and stronger TNFR1 and TNFR2 immunoreactions were observed than what was the case in the nerve fascicles described above (Figures 2D, 2E). Parts of these nerve fascicles were thus clearly immunolabelled for TNFR1 and TNFR2. There was to some extent partly an immunoreaction for TNF-α (Figure 2C).

The specimens containing distinct nerve fascicles were extensivey investigated concerning reactions for the TNF receptors, via evaluations of several sections and evaluations of additional specimens from the patients. It was found that nerve fascicles exhibiting varying degrees of TNF receptor reactions could be seen in specimens of all 11 patients for which distinct nerve fascicles were identified. As described
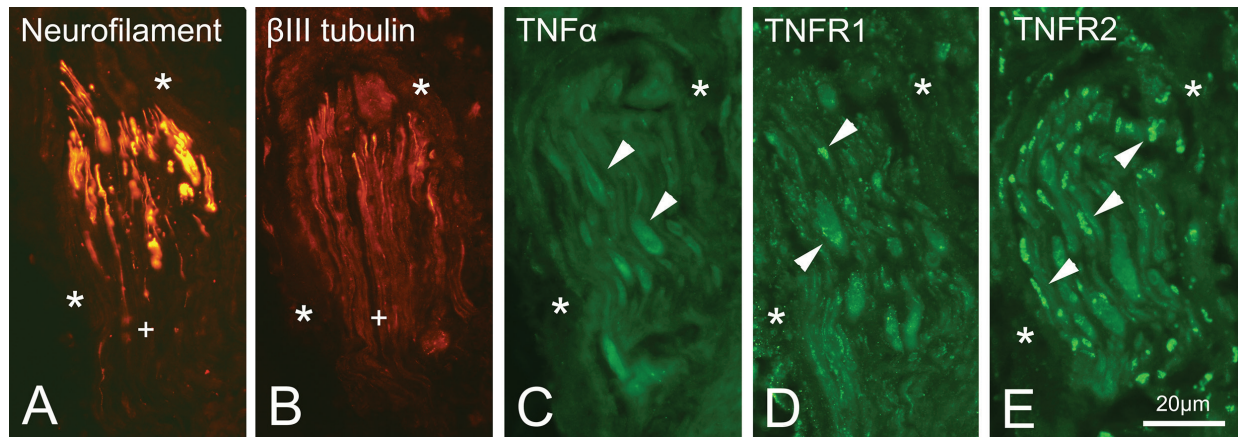
Figure 2. Serially sectioned nerve fascicle located in the connective tissue from specimen of tennis elbow patient. Large parts of the nerve fascicle are not labelled in (A) and (B), i.e. lack reactive axons (the situation is very different from that for the nerve fascicle in Fig 1). Symbols (+) in non-reactive parts of the nerve fascicle. There are certain immunoreactions (arrowheads) in (C) (staining for TNF-α). This is also the fact in (D) and particularly in (E) (arrowheads). The specific immunoreactions in (C) are weak, those in (D) and (E) strong. There is a lot of marked reactions in (E). Asterisks at corresponding sites of perineurium.
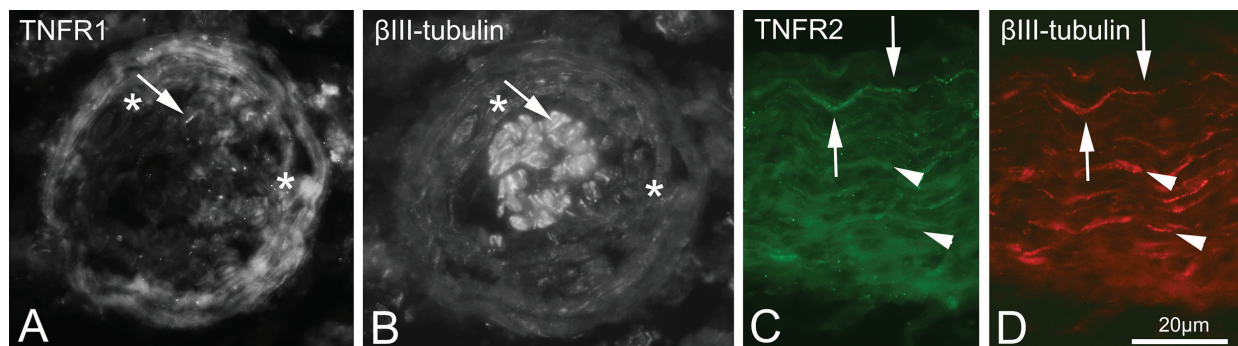

Figure 3. Cross sectioned nerve fascicle in (A,B), parts of a longitudinally cut nerve fascicle in (C,D) located in peritendinous tissue of tennis elbow region. Double stainings for TNFR1/βIII-tubulin (A,B) and for TNFR2/βIII-tubulin (C,D). Asterisks in the inner part of the perineurium in (A,B). There are βIII-tubulin immunoreactions in the nerve fascicle; only occasional TNFR1 immunoreactions are seen in the βIII-tubulin labelled axons (arrows A,B). In (D), βIII-tubulin reactive axons are seen; TNFR2 immunoreaction is observed in only a few of these (arrows). Arrowheads indicate axons where there is no co-localization. The are autofluorescence reactions in the nerve fascicle (B) and in the outer part of perineurium (A) and there are certain point-like autofluorescence reactions in (A,C).

above, the specimens also contained nerve fascicles that in principle were devoid of such reactions.

It was found relevant to understand if presence of abnormal nerve fascicles, i.e. nerve fascicles being nonhomogenously stained for βIIItubulin/neurofilament (and showing reactions for TNF receptors), was correlated to special features concerning VAS/VISA-A scores and symptom duration for the patients. It was found that symptom duration varied extensively, from approximately 3 months up to 7 years, for

this subgroup of patients. The VAS/VISA-A scores were in the magnitude of those for the entire patient population.

In order to further clarify the reaction patterns for the nerve fascicles, double-stainings for TNF-α, TNFR1 or TNFR2 and βIII-tubulin or S-100β were performed. The double-stainings showed that TNFR1 (Figure 3A) as well as TNFR2 (Figure 3C) were found to be co-localized with βIII-tubulin, i.e. the TNF receptor reactions were confined to some axons (Figures 3B, 3D). However, this was only observed to a very
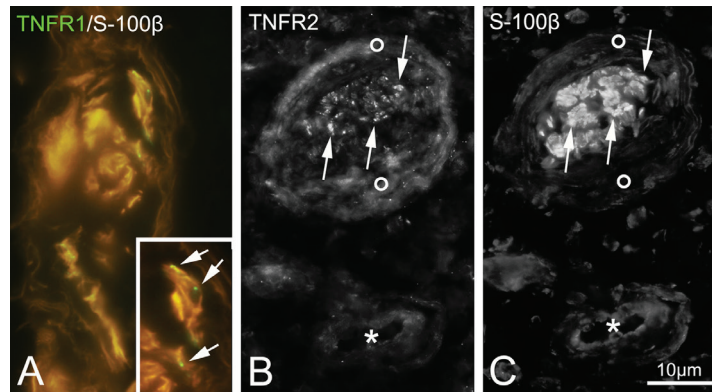
Figure 4. Peritendinous tissue; tennis elbow region. Parts of nerve fascicle in (A), cross-sectioned nerve fascicle in (B,C). Double stainings TNFR1/S-100β (A) and TNFR2/S-100β (B,C) (merged reactions in A). TNFR1 immunoreaction in green and S-100β immunoreaction in yellowish/red in (A). Inset below in (A) shows parts of (A) in higher magnification. There are TNFR1 reactions within S-100β reactive structures (arrows), i.e. within Schwann cells. In (B,C) it can be seen that TNFR2 immunoreactions occur within S-100β labelled structures (Schwann cells) (arrows). Perineurium marked with rings. Asterisks in lumen of blood vessel. There are some autofluorescent dots in (B).
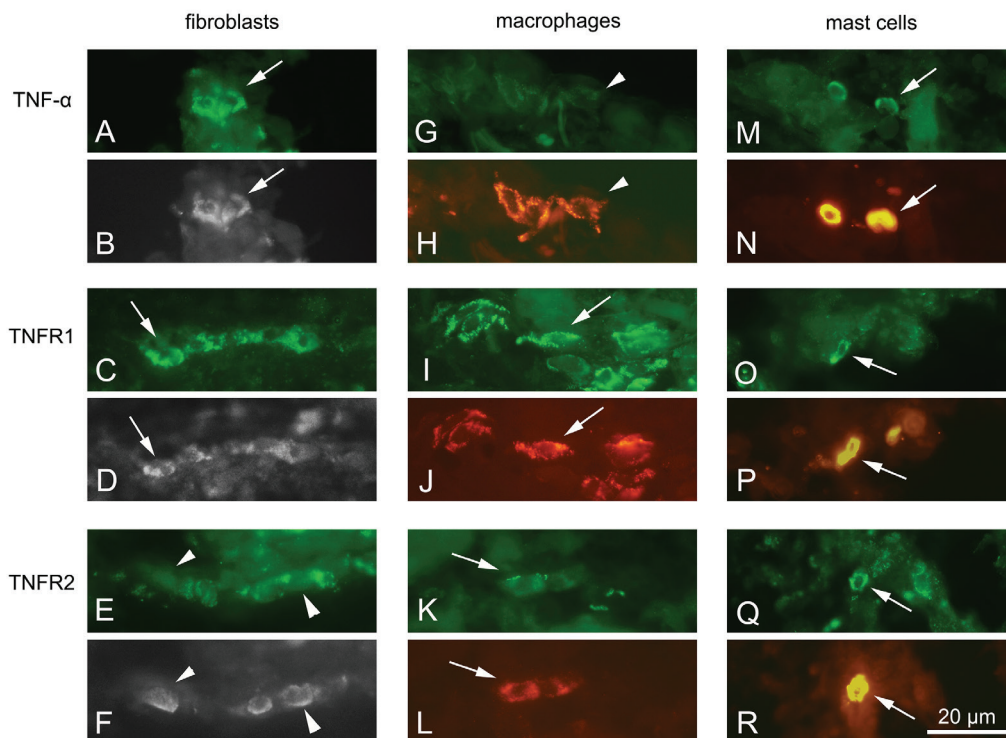

Figure 5. Double stainings showing the expression patterns for cells displaying TNFα, TNFR1 and TNFR2 immunoreactions in the peritendinous tissue (areas just outside plantaris tendons). Reactions for fibroblast marker in white colour (B,D,F), reactions for macrophage marker (CD68) in yellowish/red colour (H,J,L) and mast cell reactions in yellowish/red colour (N,P,R). TNFα, TNFR1 but not TNFR2 immunoreactions are seen in fibroblasts (A-F). Macrophages do not exhibit TNFα immunoreaction (G,H), but display TNFR1 immunoreaction (I,J) and to a certain extent TNFR2 immunoreaction (K,L). Mast cells exhibit TNFα immunoreaction (M,N) and can also be seen to show TNFR1 (O,P) and TNFR2 (Q,R) immunoreactions. Arrows indicate where colocalizations are seen, arrowheads where they are not seen.
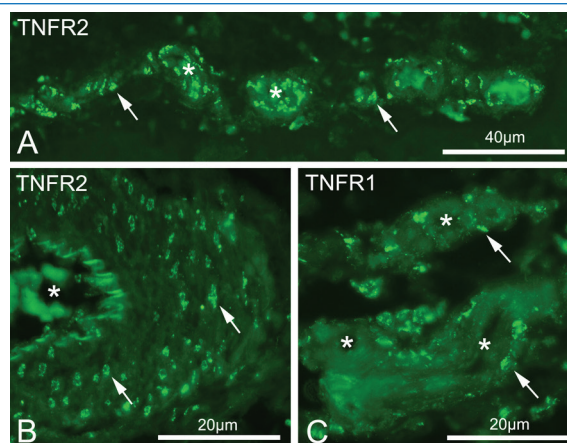
Figure 6. Peritendinous tissue in plantaris tendon specimens. There is TNFR2 immunoreaction (arrows) in the outer parts of the smooth muscle layer of a small arteriole (A). In (B), TNFR2 immunoreactions (arrows) are seen within the smooth muscle of a large arteriole. Asterisks in lumina. In (C), TNFR1 immunoreaction (arrows) is observable in the outer parts of the smooth musculature of small arterioles (asterisks in lumina). Autofluorescence reactions in inner parts of vessels.

small extent. TNFR1 (Figure 4A) and particularly TNFR2 (Figure 4B) were more frequently seen within S-1OOβ stained structures, i.e. Schwann cells. No clear pattern was observed concerning TNF-α.

Dispersed cells

There were dispersed cells in the peritendinous tissue. They conformed to white blood cells, macrophages being in majority, and fibroblasts. White blood cells and fibroblasts were observed in the connective tissue spaces of both types of specimens but were especially frequent in plantaris specimens. Immunoreactions for TNFR1 and TNFR2 were very frequently seen in the dispersed cells, and to a lesser extent TNF-α immunoreactions were seen in these. Double-stainings for TNF-α and TNF receptors in relation to the various markers for the cells were performed.

Fibroblasts: There was a very frequent co-localization with reactions for TNF-α (Figures 5A, 5B) in the cells depicted by the fibroblast marker. TNFR1 immunoreactions also frequently coexisted with reactions for this marker (Figures 5C, 5D). On the other hand, there was usually a non-existence of colocalization between immunoreactions for TNFR2 and fibroblast marker (Figures 5E, 5F).

Macrophages: The macrophages, visualized via staining for CD68, did never exhibit TNF-α immunoreaction (Figures 5G, 5H). TNFR1 was on the other hand very frequently observed for these cells (Figures 5I, 5J). TNFR2 was expressed by some macrophages (Figures 5K, 5L).

Mast cells: The mast cells often showed immunoreaction for TNF-α (Figures 5M, 5N) and did to some extent also exhibit TNFR1 (Figures 5O, 5P) and TNFR2 (Figures 5Q, 5R) immunoreaction.

Other white blood cells: Stainings for eosinophils and stainings by using the T-cell/neutrophil antibody were made. Immunoreactions for these markers were very infrequently seen in the dispersed cells in the specimens. Co-localization with immunoreactions for TNF-α, TNFR1 and TNFR2 was never seen for any of these cell types.

Blood vessels

Arterioles, venules and fine blood vessels were seen in the peritendinous tissue of both plantaris and tennis elbow specimens. Some of these vessels in the former tissue were seen to abut the tendon tissue proper of the plantaris tendons.

The blood vessel walls were seen to express marked reactions for TNFR2 (Figure 6A). The reactions were located to the smooth muscle layer and were not seen for the endothelium. The reactions were mainly confined to arterioles of small dimensions and were here located in the outer parts of the smooth muscle layer (Figure 6A). TNFR2 immunoreactions were sometimes seen in the walls of large arterioles as well (Figure 6B). Immunoreactions for TNFR1 were also seen in the arteriolar walls, but to a lesser extent than those seen for TNFR2 (Figure 6C). It was on the whole apparent that TNFR2/TNFR1 immunoreactions occurred for some of the blood vessels in the peritendinous tissue, whilst others were non-reactive. There were weak immunoreactions for TNF-α in parts of the small blood vessels.

Discussion

The TNF-α system is here shown to be expressed in nerve fascicles, blood vessel walls and dispersed cells in the peritendinous tissue in tendinopathy/tennis elbow patients.

The observations suggest that these structures can be driven by the TNF-α system.

Findings for the nerve fascicles

The findings for the nerve fascicles were especially interesting. Due to ethical considerations, it was not possible to obtain peritendinous tissue of ECRB/common extensor origin and plantaris regions via operations on completely healthy individuals. Nevertheless, it was obvious that differences occurred in the structure of the existing nerve fascicles in the operated patients. There were thus some that displayed a partial absence of axons, as seen via stainings for βIII-tubulin and neurofilament. This observation is in accordance with previous findings for the peritendinous tissue of the Achilles/plantaris tendon region[7] suggesting that a partial axonal degeneration has occured. It is thus possible that there are tissue influences in the peritendinous tissue that to some extent are harmful for the nerve fascicles in the tendinopathy/tennis elbow situations.

Our studies showed that there were clear expressions for TNFR1 and TNFR2, and to a lesser extent also for TNF-α, in the nerve fascicles exhibiting a partial loss of axons. The magnitude of expressions were much lower in the nerve fascicles having a normal appearance. It seems as if the harmful effects on the nerves in the tendinopathy/tennis elbow situations lead to upregulations of the TNF-α system for the innervation. It can be asked as to whether this is related to the pain sensations that prevail in the peritendinous tissue. What is known is that TNF-α can be a mediator of pain[12,34]. Furthermore, scraping of the peritendinous tissue on the ventral side of the Achilles tendon on patients with Achilles tendinopathy have shown very good clinical results with significantly lowered pain and discomfort during Achilles tendon loading activity[4]. It is also known that anti-TNF treatment inhibits pain response in the CNS, as seen via functional MRI evaluations[34].

One possibility is that TNF-α is involved in the nerve degeneration that occurs in the nerve fascicles. Accordingly, TNF-α has in other situations been shown to induce degenerative features for neurons[35]. Comparisons can be made with other situations leading to nerve damage. There is one report saying that there is an increased immunofluorescence for TNFR1, as well as TNF-α, in dorsal root ganglia after operations leading to nerve injury[36]. It has also been shown that there is an upregulation of TNFR1 expression in dorsal root ganglion neurons in response to lumbar facet joint injury[37]. Nevertheless, it is known that TNF-α can be responsible for not only induction of degenerative features but also attempts for neurite outgrowth[38].

Double-stainings showed that the TNFR1 and TNFR2 reactions were only sometimes detectable for axonal profiles but that they, especially the TNFR2 reactions, were more clearly detectable for Schwann cells. The findings are in accordance with observations that TNFR1 and TNFR2 receptors can be present in both neurons and glia cells[39,40]. It is well-known that TNFR1 and TNFR2 have distinct ligand-binding affinities and that they elicit different physiological functions, not least in relation to effects on nerves[39]. Most evidence shows that TNFR1 signaling mediates deleterious pro-inflammatory, cytotoxic and damaging effects whilst signaling via TNFR2 is related to neuroprotection, proliferation signaling and repair mechanisms[41,42]. Concerning our findings of marked TNFR2 reactions for Schwann cells it should be remembered that TNF-α in other situations is suggested to stimulate Schwann cell proliferation[43]. It can not be excluded that TNFR2 has an essential role in neuroprotection for the nerve fascicles in the peritendinous tissue. Establishments of such effects via TNFR2 have been suggested to be effective approaches in the treatment of neurodegenerative diseases[44]. As seen in ischemia reperfusion studies, TNFR2 signalling is reported to be required for survival of retinal neurons[45].

Findings for blood vessels and dispersed cells

We found that there was a marked expression of TNFR1 and especially TNFR2 in the walls of certain of the blood vessels. TNF-α can thus be involved in driving a blood vessel remodeling process in the tissue, a function that is ascribed the TNF-α system for the blood vessels in inflammation processes[17]. The receptor reactions were confined to the smooth muscle layer. Of interest in this respect is that TNF-α can mediate the proliferation of vascular smooth muscle cells[46]. In the upregulation for the TNF-α system in response to subarachnoid hemmorhage, the primary localizations for the immunoreactivities for the TNF receptors were found to be the cell membrane and the cytoplasm of the smooth muscle cells[18]. Of interest is also the observation that the TNF-α system is upregulated in ischemia. Thus, retinal ischemia results in increased expression of TNF-α as well as TNFR1 and TNFR2[19].

We observed that the cells that were frequently present in the peritendinous tissue mainly conformed to macrophages and fibroblasts but that some of them were mast cells. In accordance with our findings concerning expressions of the elements in the TNF-α system it is well-known that both TNFR1 and TNFR2 are known to be expressed on macrophages[8] and that mast cells can produce TNF-α[41]. TNF-α is furthermore known to promote macrophage differentiation and is shown to promote proliferation of fibroblasts[47,48].

The TNF-α produced by the cells in the peritendinous tissue can actually elicit autocrine effects, as is suggested to be the case for locally produced TNF-α in CNS disorders[49]. The peritendinous macrophages, being equipped with TNFR1 and TNFR2 receptors, can hereby have a special importance as these cells are reported to be an essential component in the regenerative phase in tissue regeneration upon damage[50]. A tisse for which this effect is reported is muscle tissue. It is thus suggested that TNF-α modulates the regenerative process in the damaged muscle tissue[51].

It has long been debated as to whether there are inflammation features in tendinopathy or if the condition is entirely related to degenerative changes[52]. In the present study we show that there is a large number of white blood

cells, especially macrophages, in the peritendinous tissue in the tendinopathy situation. One suggestion that is previously put forward is that inflammation and degeneration are not mutually exclusive but that they work together in the pathogenesis of tendinopathy[53]. The frequent presence of white blood cells in the peritendinous tissue may be of great importance in the reorganization/regenerating process in the tissue.

Concluding remarks

The peritendinous tissue is a tissue which represents a dynamic and responsive region[54] and a tissue in which the concentrations of substances such as matrix metalloproteinases and their tissue inhibitors are increased in response to physical exercise[55]. The findings in the present study suggest that TNF-α has effects in this tissue in tendinopathy/tennis elbow and that these effects can be related to damaging features as well as attempts for healing.

It has previously been suggested that pro-inflammatory cytokines such as TNFalpha can be mediators in the healing of ruptured tendons[56]. Although the tendon cells themselves can produce TNF-α[20], an important source might, apart from fibroblasts in the tissue, be blood-derived white blood cells that are attracted to the peritendinous tissue. Presence of white blood cells in the peritendinous tissue during healing in a rat tendon rupture method has actually been noted[57]. As is discussed above, it is possible that the frequently occurring macrophages expressing TNF receptors in the peritendinous tissue are related to attempts for tissue regeneration. The findings concerning TNF receptor expressions in the nerve fascicles showing deficits of axons can be related to both attempts for neurite outgrowth and to nerve degeneration.

A limitation of the study is that specimens of completely healthy indivicuals could not, due to ethical considerations, be evaluated.

In conclusion, the cytokine TNF-α and its receptors are much expressed in the peritendinous tissue why this cytokine can have a great impact in this tissue in tendinopathy/tennis elbow.

Author contributions

All authors were involved in the design of the study, the interpretation of the results and the drafting of the manuscript. CS processed the tissue specimens, performed analyses and was responsible for creating the figure montages. LR conducted analyses and was involved in conducting figure montages. HA performed all operations and was responsible the delivery of the tissue. SF performed analyses, wrote the first draft of the manuscript and made the conception of the figures. All authors approved the final version of the manuscript.

References

1. Kjaer M, Bayer ML, Eliasson P, et al. What is the impact of inflammation on the critical interplay between mechanical signaling and biochemical changes in tendon matrix? J Appl Physiol 2013;115:879-83.
2. Heinemeier KM, Schjerling P, Heinemeier J, et al. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C. FASEB J 2013;27:2074-9.
3. Andersson G, Danielson P, Alfredson H, et al. Nerve-related characteristics of ventral paratendinous tissue in chronic Achilles tendinosis. Knee Surg Sports Traumatol Arthrosc 2007;15:1272-9.
4. Alfredson H. Ultrasound and Doppler-guided mini-surgery to treat midportion Achilles tendinosis: results of a large material and a randomised study comparing two scraping techniques. Br J Sports Med 2011;45:407-10.
5. Alfredson H, Öhberg L, Zeisig E, et al. Treatment of midportion Achilles tendinosis: similar clinical results with US and CD-guided surgery outside the tendon and sclerosing polidocanol injections. Knee Surg Sports Traumatol Arthrosc 2007;15:1504-9.
6. Spang C, Alfredson H, Ferguson M, et al. The plantaris tendon in association with mid-portion Achilles tendinosis - Tendinosis-like morphological features and presence of a non-neuronal cholinergic system. Histol Histopathol 2013;28:623-32.
7. Spang C, Harandi VM, Alfredson H, et al. Marked innervation but also signs of nerve degeneration in between the Achilles and plantaris tendons and presence of innervation within the plantaris tendon in midportion Achilles tendinopathy. J Musculoskeletal Neuronal Interact 2015;15:197-206.
8. Ermert E, Pantazis C, Duncker H-R, et al. *In situ* localization of TNFalpha/beta, TACE and TNF receptors TNF-R1 and TNF-R2 in control and LPS-treated lung tissue. Cytokine 2003;22:89-100.
9. Takei Y, Laskey R. Tumor necrosis factor alpha regulates responses to nerve growth factor, promoting neuronal cell survival but suppressing differentiation of neuroblastoma cells. Mol Biol Cell 2008;19:855-64.
10. Schafers M, Geis C, Svensson CI, et al. Selective increase of tumor necrosis factor-alpha in injured and spared myelinated primary afferents after chronic constrictive injury of rat sciatic nerve. Eur J Neurosci 2003;17:791-804.
11. Shubayev VI, Myers RR. Anterograde TNF-alpha transport from rat dorsal root ganglion to spinal cord and injured sciatic nerve. Neurosci Lett 2002;320:99-101.
12. Frauenfelder SR, Freiberger SN, Bavinck JNB, et al. Prostaglandin E2, tumor necrosis factor alpha, and pro-opiomelanocortin genes as potential mediators of cancer pain in cutaneous squamous cell carcinoma of organ transplant recipients. JAMA Dermatol Epub 2016 (ahead of print).
13. Boettger MK, Hensellek S, Richter F, et al. Antinociceptive effects of tumor necrosis factor alpha neutralization in

a rat model of antigen-induced arthritis: evidence of a neuronal target. Arthritis Rheum 2008;58:2368-78.

14. Sriram K, Matheson JM, Benkovic SA, et al. Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF-alpha. FASEB J 2006;20:670-82.

15. Camara-Lemarroy CR, Guzman-de la Garza FJ, Fernandez-Garza NE. Molecular inflammatory mediators in peripheral nerve degeneration and regeneration. Neuroimmunomodulation 2010;17:314-324.

16. Dinarello CA. The interleukin-1 family: 10 years of discovery. FASEB J 1994;8:1314-25.

17. Baluk P, Yao LC, Feng J, et al. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. J Clin Invest 2009;119:2954-64.

18. Maddahi A, Kruse LS, Chen QW, et al. The role of tumor necrosis factor-alpha and TNF-alpha receptors in cerebral arteries following cerebral ischemiain rat. J Neuroinflammation 2011;8:107.

19. Gesslein B, Håkansson G, Gustafsson L, et al. Tumor necrosis factor and its receptors in neuroretina and retinal vasculature after ischemia-reperfusion injury in the pig retina. Mol Vis 2010;16:2317-27.

20. Gaida JE, Bagge J, Purdam C, et al. Evidence of the TNF-α system in the human Achilles tendon: expression of TNF-α and TNF receptor at both protein and mRNA levels in the tenocytes. Cells Tissues Organs 2012;196:339-52.

21. Alfredson H. Midportion Achilles tendinosis and the plantaris tendon. Br J Sports Med 2011;45:1023-5.

22. Masci L, Spang C, van Schie HT, et al. How to diagnose plantaris tendon involvement in midportion Achilles tendinopathy - clinical and imaging findings. BMC Musculoskelet Disord 2015;17:97.

23. Spang C, Alfredson H. Richly innervated soft tissues covering the superficial aspect of the extensor origin in patients with chronic painful tennis elbow - Implication for treatment? J Musculoskelet Neuronal Interact 2017;17:97-103.

24. Masci L, Spang C, van Schie HT, et al. Achilles tendinopathy - do plantaris tendon removal and Achilles tendon scraping improve tendon structure? A prospective study using ultrasound tissue characterisation. BMJ Open Sp Ex Med 2015;1:e000005.

25. Smith J, Alfredson H, Masci L, et al. Differential Plantaris-Achilles tendon motion: A sonographic and cadaveric investigation. PM R 2016; Epub, ahead of print.

26. Spang C, Alfredson H, Docking SI, et al. The plantaris tendon: a narrative review focusing on anatomical features and clinical importance. Bone Joint J 2016;98-B:1312-9.

27. Forsgren S, Renström L, Purdam C, et al. TNF-Alpha in the Locomotor System beyond Joints: High Degree of Involvement in Myositis in a Rabbit Model. Int J Rheumatol 2012;637452.

28. Pettersson J, Kalbermatten D, McGrath A, et al. Biodegradable fibrin conduit promotes long-term regeneration after peripheral nerve injury in adults rats. J Plast Reconstr Aesthet Surg 2010;63:1893-9.

29. Tse KH, Sun M, Mantovani C, Terenghi G, et al. In vitro evaluation of polyester-based scaffolds with adipose derived stem cells in peripheral nerve regeneration. J Biomed Mater res A 2010;95:701-8.

30. McGrath AM, Brohlin M, Kingham PJ, et al. Fibrin conduit supplemented with human mesenchymal stem cells and immunosuppresiive treatment enhances regeneration after peripheral nerve injury. Neurosci Lett 2012;516:171-6.

31. Song Y, Stål PS, Yu J, et al. Marked Effects of Tachykinin in Myositis Both in the Experimental Side and Contralaterally: Studies on NK-1 Receptor Expressions in an Animal Model. ISRN Inflammation 2013;907821.

32. Spang C, Scott A, Danielson P, et al. VGluT2 and NMDAR1 expression in cells in the inflammatory infiltrates in experimentally induced myositis: evidence of local glutamate signaling suggests autocrine/paracrine effects in an overuse injury model. Inflammation 2012;35:39-48.

33. Hansson M, Forsgren S. Immunoreactive atrial and brain natriuretic peptides are co-localized in Purkinje fibres but not in the innervation of the bovine heart conduction system. Histochem J 1995;27:222-30.

34. Hess A, Axmann R, Rech J, et al. Blockade of TNF-α rapidly inhibits pain responses in the central nervous system. Proc Natl Acad Sci USA 2011;108:3731-6.

35. Tezel G. TNF-alpha signaling in glaucomatous neurodegeneration. Prog Brain Res 2008;173:409-21.

36. Dubovy P, Jancalek R, Klusakova I, et al. Intra- and extraneuronal changes of immunofluorescence staining for TNF-alpha and TNFR1 in the dorsal root ganglia of rat peripheral neuropathic pain models. Cell Mol Neurobiol 2006;26:1205-17.

37. Sakuma Y, Ohtori S, Miyagi M, et al. Up-regulation of p55 TNF alpha-receptor in dorsal root ganglia neurons following lumbar facet joint injury in rats. Eur Spine J 2007;16:1273-8.

38. Schmitt KR, Boato F, Diestel A, et al. Hypothermia-induced neurite outgrowth is mediated by tumor necrosis factor-alpha. Brain Pathol 2010;20:771-9.

39. Vandenabeele P, Declercq W, Beyaert R, et al. Two tumor necrosis factor receptors: structure and function. Trends Cell Biol 1995;5:392-9.

40. Morganti-Kossman MC, Lenzlinger PM, Hans V, et al. Production of cytokines following brain injury: beneficial and deleterious for the damaged tissue. Mol Psychiatry 1997;2:133-6.

41. Ohkawara Y, Yamauchi K, Tanno G, et al. Human lung mast cells and pulmonary macrophages produce tumor necrosis factor-alpha in sensitized lung tissue after IgE receptor triggering. Am J Respir Cell Mol Biol 1992;7:385-92.

42. Probert L. TNF and its receptors in the CNS: the essential, the desirable and the deleterious effects. Neuroscience 2015;302:2-22.

43. Mosnier F-F, Jarry A, Camdessanche J-P, et al. *In situ* evidence of involvement of Schwann cells in ulcerative colitis: autocrine and paracrine signaling by A disintegrin and metalloproteas-17-mediated tumor necrosis factor alpha production. Human Pathology 2009;40:1159-67.

44. Dong Y, Fischer R, Naude PJ, et al. Essential protective role of tumor necrosis factor receptor 2 in neurodegeneration. Proc Natl Acad Sci USA 2016; 113:12304-9.

45. Fontaine V, Mohand-Said S, Hanoteau N, et al. Neurodegenerative and neuroprotective effects of tumor necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. J Neurosci 2002;22:RC216.

46. Qi L, Zhi J, Zhang T, et al. Inhibition of microRNA-25 by tumor necrosis factor alpha is critical in the modulation of vascular smooth muscle cell proliferation. Mol Med Rep 2015;11:4353-4358.

47. Trinchieri G, Kobayashi M, Rosen M, et al. Tumor necrosis factor and lymphotoxin induce differentaition of human myelid cell lines in synergy with immune interferon. J Exp Med 1986;164:1206-25.

48. Palombella VJ, Mendelsohn J, Vilcek J. Mitogenic action of tumor necrosis factor in human fibroblasts: interaction with epidermal growth factor and platelet-derived growth factor. J Cell Physiol 1988;135:23-31.

49. Kuno R, Wang J, Kawanokuchi J, et al. Autocrine activation of microglia by tumor necrosis factor-alpha. J Neuroimmunol 2005;162:89-96.

50. Tidball JG, Villata SA. Regulatory interactions between muscle and the immune system during muscle regeneration. Am J Physiol Regul Integr Comp Physiol 2010;298:R1173-87.

51. Warren GL, Hulderman T, Jensen N, et al. Physiological role of tumor necrosis factor alpha in traumatic muscle injury. FASEB J 2002;16:1630-2.

52. Kaux J-F, Forthomme B, Le Goff C, et al. Current opinions in tendinopathy. J Sports Science Med 2011;10:238-253.

53. Abate M, Silbernagel KG, Siljeholm C, et al. Pathogenesis of tendinopathies: inflammation or degeneration? Arthritis Res Ther 2009;11:235.

54. Kjaer M, Langberg H, Skovgaard D, et al. *In vivo* studies of peritendinous tissue in exercise. Scand J Med Sci Sports 2000;10:326-331.

55. Koskinen SO, Heinemeier KM, Olesen JL, et al. Physical exercise can influence local levels of matrix metalloproteinases and their inhibitors in tendon-related connective tissue. J Appl Physiol 2004;96:861-864.

56. Schulze-Tanzil G, Al-Sadi O, Wiegand E, et al. The role of pro-inflammatory and immunoregulatory cytokines in tendon healing and rupture: new insights. Scand J Med Sci Sports 2011;21:337-51.

57. Wojciak B, Crossan JF. The accumulation of inflammatory cells in synovial sheath and epitenon during adhesion formation in healing rat flexor tendons. Clin Exp Immunol 1993;193:108-114.