Different gene response to mechanical loading during early and late phases of rat Achilles tendon healing

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Title:

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Gene response after loading in healing tendons

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Abstract

Mechanical loading stimulates tendon healing both when applied in the inflammatory phase and in the early remodeling phase of the process, although not necessarily via the same mechanisms. We investigated the gene response to mechanical loading in these two phases of tendon healing. The right Achilles tendon in rats was transected and the hind limbs were unloaded by tail suspension. The rats were exposed to 5 minutes of treadmill running 3 or 14 days after tendon transection. Thereafter, they were re-suspended for 15 minutes or 3 hours until euthanasia. The controls were suspended continuously. Gene analysis was first performed by microarray analysis followed by qRT-PCR on selected genes, focusing on inflammation. 15 minutes after loading, the most important genes seemed to be the transcription factors EGR1 and C-FOS, regardless of healing phase. These transcription factors might promote tendon cell proliferation and differentiation, stimulate collagen production, and regulate inflammation. 3 hours after loading Day 3, inflammation was strongly affected. Seven inflammation-related genes were up-regulated according to PCR; CCL20, CCL7, IL-6, NFIL3, PTX3, SOCS1, and TLR2. These genes can be connected to macrophages, T cells, and recruitment of leukocytes. According to Ingenuity pathway analysis, the recruitment of leukocytes was increased by loading Day 3 which also was confirmed by histology. This inflammation-related gene response was not seen Day 14. Our results suggest that the immediate gene response after mechanical loading is similar in the early and late phases of healing, but the late gene response is different.

New & Noteworthy

This study investigates the direct effect of mechanical loading on gene expression during different healing phases in tendon healing. One isolated episode of mechanical loading was studied in otherwise unloaded healing tendons. This enabled us to study a time sequence, i.e. which genes were the first ones to be regulated after the loading episode.

Introduction

Tendons adapt to mechanical loading (25, 31, 39, 47, 60). Tenocytes register mechanical load or deformation via mechanotransduction, leading to changes in gene and protein expression (11, 13, 25, 31). The changes can lead to increased matrix production, including increased expression of collagens, thus enabling the tendon to cope with increased loading (13, 25, 31, 47, 60).

Mechanical loading is also important during tendon healing (3, 7, 25, 31, 47, 52). A loaded healing tendon becomes several-fold stronger, with better material properties, compared to unloaded healing tendons (2, 3, 22). As little as 5 minutes of daily mechanical stimulation is sufficient to improve Achilles tendon healing in rats (23). The underlying mechanisms have not been clarified. However, mechanical loading induces changes in gene expression in healing tendons (7, 22, 31). We have previously shown that a single loading episode in an otherwise unloaded healing tendon, 5 days after injury, alters the gene expression pattern (23). 5 genes were up-regulated already 15 minutes after loading, and 4 of these were transcription factors. Interestingly, one of the transcription factors, early growth response 1 (EGR1), has been shown to be important for tendon development and promotes tendon repair (26, 28, 34). 3 hours after loading, 150 genes were regulated, including genes involved in inflammation, wound healing, apoptosis, cell proliferation, cell differentiation and extracellular matrix remodeling (21).

Tendon healing can be divided into three overlapping phases; inflammation, proliferation and remodeling (25, 31). Contrary to what has been believed, mechanical loading has a stimulatory effect not only during the early remodeling phase but also when applied during the inflammatory phase (2, 20). Although beneficial in both phases, the mechanism might be different, because the

cellular and matrix composition of the loaded region is different (25, 31, 41). In the inflammatory phase, the healing tissue contains a high proportion of inflammatory cells, and a loose fibrous stroma, rich in collagen type III (6, 25, 31, 41). Later, in the early remodeling phase there is a denser connective tissue dominated by collagen type I together with fibroblast or tenocytes and few inflammatory cells (6, 25, 31, 41).

To better understand how mechanical loading improves tendon healing, we now studied the gene expression in healing tendons during different phases: the inflammatory phase and the early remodeling phase. To understand the specific effects of loading, we studied isolated episodes of loading in otherwise unloaded tendons, which enables us to study a time sequence of regulated genes. In contrast, a comparison of animals that have been continuously loaded or unloaded would just have shown the gene expression pattern far down-stream of loading, i.e. genes that are associated with the consequences of loading, such as a stronger and thicker tendon.

This report describes the gene expression pattern after a single loading episode in the inflammatory and early remodeling phase of tendon healing, i.e. 3 or 14 days after injury, respectively. The immediate gene response, 15 minutes after loading, and the late gene response, 3 hours after loading, were studied using microarray and quantitative real-time PCR (qRT-PCR). The general hypothesis was that the response to loading would be different during different phases of the healing process, especially regarding inflammation-related genes.

Materials and Methods

Study design

82 female Sprague-Dawley rats (10-12 weeks old) were used for this study; 24 for microarray analyses (N=4), 33 for qRT-PCR analyses (N=4-7), and 8 for histology (N=4). 17 rats had to be excluded; 12 because they had fallen down from the tail suspension device, 4 because they had chewed on their wounds, and 1 rat was excluded because the RNA extraction failed.

The right Achilles tendon of all rats was transected, and allowed to heal spontaneously. All rats were unloaded by tail suspension the day after transection. The rats were killed 3 or 14 days after transection. The controls were tail suspended continuously until euthanasia. The rats to be stimulated by mechanical loading were taken down from suspension and exposed to a single 5 minutes episode of loading by treadmill running on the day of euthanasia. For gene expression analyses, the rats were re-suspended for 15 minutes or 3 hours until they were killed for tissue harvesting. For histology, the rats were re-suspended for 3 hours until they were killed for tissue harvest (Figure 1).

All rats were randomized to the different groups by a lottery. Blinded evaluation was ensured by the fact that the samples had an identity number which could not be connected to a specific group during the whole analysis.

All experiments were approved by the Regional Ethics Committee in Linköping for animal experiments and adhered to the institutional guidelines for care and treatment of laboratory animals. The rats were housed one per cage and were given food and water *ad libitum*.

Surgery

All rats were anesthetized with isoflurane gas (Forene, Abbot Scandinavia, Solna, Sweden) and given antibiotics (25 mg/kg, Oxytetracycline, Engemycin; Intervet, Boxmeer, The Netherlands) preoperatively. They were also given analgesics (0.045 mg/kg Buprenorphine, Temgesic;

Schering-Plough, Brussels, Belgium) preoperatively and regularly until 48 hours after surgery.

The surgery was performed under aseptic conditions. The skin over the right Achilles tendon was shaved and washed with chlorhexidine ethanol. A transverse skin incision was made lateral to the Achilles tendon, and the tendon complex was exposed. The plantaris tendon was removed.

The Achilles tendon was transversely transected, and a 3 mm-segment was removed. The tendon was left to heal spontaneously without any sutures. The skin was closed by two stitches.

Unloading

One day after surgery, the hind limbs of all rats were unloaded by tail suspension. The tail suspension was carried out in special cages with an overhead system that allowed the rats to rotate and move in all directions using their forelimbs, whereas the hind limbs were lifted just above the cage floor. The rats had been acclimatized to the suspension cages before surgery. For suspension, an adhesive tape was attached to the rat's tail. The tape was connected to the overhead system by a fish-line swivel and a fish line. The tail suspension technique is described in more detail elsewhere (46).

Mechanical loading

The animals were either kept completely unloaded for the entire experiment or were subjected to a short, single loading episode, 3 or 14 days after tendon transection. During the loading episode,

the rats were released from the suspension to walk on a treadmill for 5 minutes at 9 m/min, 7.5° uphill slope, before they were suspended again. The animals were monitored during the loading episode.

Tissue harvesting

66 rats were anesthetized with a subcutaneous injection (0.5 mg/kg dexdomitor; Orion Pharma, Espoo, Finland; and Ketaminol, Intervet) while still suspended, and were taken down first when they were fully sedated. The corresponding unloaded controls were killed at the same time points. 58 rats were used for RNA extraction and harvested as follows during sedation. The skin was shaved and washed, and the healing tissue was dissected free of the surrounding soft tissue under aseptic conditions. Tissue harvest was timed to either 15 minutes or 3 hours after loading. A midpart segment from the healing tendon (consisting of newly formed tissue only) was harvested, quickly rinsed in sodium chloride, snap-frozen in liquid nitrogen, and stored at -70°C until RNA extraction. The rats were thereafter euthanized with an overdose of pentobarbital sodium. The hind limbs were kept unloaded throughout the whole harvesting procedure. 8 rats were used for histology and harvested as follows, after euthanasia with carbon dioxide. The skin was shaved and the healing tissue was dissected free from the surrounding soft tissue. The healing Achilles tendon was taken out together with a small piece of the calf muscle (for orientation of the tissue) and placed on a silicone gel with two needles in purpose to keep the tendon straight.

RNA extraction

Extraction of RNA was carried out by a combination of the Trizol method and RNeasy total mini kit (QIAGEN, Sollentuna, Sweden). The tendons were kept frozen during homogenization and were pulverized one by one by a tungsten ball in liquid nitrogen– cooled vessels in a Retsch mixer mill MM 200 (Retsch, Haan, Germany). Trizol was added to the pulverized tendons and left to thaw at room temperature. Chloroform was added to the samples, followed by centrifugation and phase separation. The aqueous phase was transferred to new tubes containing 70% ethanol. RNA was further purified using the RNeasy total mini kit according to the manufacturers' instructions. Potential DNA contamination was eliminated by DNase treatment. RNA concentration and quality were analyzed with the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA 6000 Nano kit (Agilent Technologies, Böblingen, Germany). RNA samples were stored at -70°C until used.

Microarray

4 samples from each group were analyzed by rat microarrays (GeneTitan, Gene ST 1.1; Affymetrix, Santa Clara, CA). The microarray (and the statistical analysis of the microarray) was carried out by the Bioinformatics and Expression Analysis (BEA) core facility at the Karolinska Institute, Stockholm. Differentially regulated genes were based on both fold change and the p-value from a Student's t-test (RMA analyzed data) with a cutoff of ≥ 1.5 or ≤ -1.5 for fold change together with a p-value of < 0.05 . The Ingenuity Pathway Analysis software was used to assess the molecular and cellular functions from the microarray results from gene data obtained 3 hours after loading on both days. Only functions with an activation z-score of ≥ 2 or ≤ -2 and predicted upstream regulators with a p-value of < 0.05 were considered.

Quantitative real-time PCR

33 new samples, 4-7 rats in each group, were used to confirm selected genes from the microarray analysis and 1.5 µg of total RNA was transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, United Kingdom). The cDNA was diluted in Tris-EDTA buffer. 46 primers were used and purchased from Applied Biosystems (Life Technologies, Netherlands; Table 1). Amplification was performed in 15 µl reactions using TaqMan Fast PCR Master mix (Life Technologies, Great Britain) and each sample was analyzed in triplicate. Quantitative RT-PCR was conducted using the $\Delta\Delta C_t$ method and each sample was normalized to 18S rRNA (Life technologies, Sweden). The down-regulated genes had a fold change between 0 and 1 and were converted to a negative value by this formula: $-1/\text{fold change}$. The efficiency for the normalizer (18S) and the target genes were all acceptable (slope ≤ 0.1). The expression of the reference gene did not vary significantly between the groups. Reactions with no reverse transcription and no template were added as negative controls.

Histology

The tendons were fixed in 4% phosphate buffered formaldehyde. After dehydration, the specimens were embedded in paraffin and sectioned parallel to the longitudinal axis of the tendon. One slide per specimen, comprising the full length of the healing tendon, was stained with Ehrlich hematoxylin and eosin. Two more slides were stained for CD45⁺ cells (leukocytes) and CD68⁺ cells (macrophages) detected by immunoperoxidase staining as follows. For antigen retrieval, citrate buffer was used for 1 h together with a high temperature (70-80 °C). Sections were washed in Tris buffered saline (TBS), incubated for 5 minutes in 3% hydrogen peroxide, washed again with TBS and 0.025% Triton X-100 followed by distilled water, and incubated for

20 minutes in Protein Block Serum-Free (Dako, USA). A polyclonal rabbit anti-rat primary antibody was used for CD45 detection (anti-CD45 antibody, ab10558, Abcam, United Kingdom) diluted 1:200 and for CD68 detection (anti-CD68 antibody, ab125212, Abcam, United Kingdom) diluted 1:200, incubated overnight in room temperature. Sections were washed in TBS, incubated for 1 h with a biotylated secondary antibody (Dako, USA) diluted 1:200, and washed again in TBS. Horseradish peroxidase (HRP)-labeled streptavidin (Vector laboratories, USA) was added for 1 h, followed by washing in TBS, and detected by 3,3'-diaminobenzidine (DAB; ACROS Organics, Belgium) substrate for 20 minutes. Finally, sections were washed in TBS and running water (3-4 minutes), counterstained with Mayers HTX (1:5) for 4 minutes, and washed in running water (3-4 minutes).

Estimation of the amount of stained cells for CD45 or CD68 was performed with a light microscope at 4x, 10x, and 20x magnifications. Each specimen was examined by two independent investigators. Both investigators graded the specimens independently from 1-3, where 3 corresponds to a high number of stained cells. The sum of the grades with the different magnifications was calculated, i.e. the grading ranges from 3-9, and then the mean was calculated from the two investigators. There was a discrepancy between the two investigators in 3 specimens (Specimen A and G for CD45 and C for CD68, Figure 3). In these cases a third investigator evaluated the specimens and decided which grade was correct. All investigators were blinded for treatment during the evaluation.

Statistical analysis

Microarray analysis results were regarded as descriptive. However, possibly regulated genes were defined by use of a p-value <0.05 as calculated by Student's t-test and a fold change of ≥ 1.5 or ≤ -1.5 . Confirmatory qRT-PCR results and histology were analyzed with Mann-Whitney U test.

Results

Day 3

EGR1 and C-FOS were immediately up-regulated

15 minutes after loading, 11 genes were regulated according to the microarray analysis Day 3 (Table 2). 3 of the genes might be involved in inflammation: Complement component 6 (C6), Ficolin B (FCNB), and Z-DNA binding protein 1 (ZBP1). For confirmation by qRT-PCR, C6 and FCNB were chosen together with 5 genes that have previously been found to be up-regulated by loading at day 5 in healing tendons, namely EGR1, Early growth response 2 (EGR2), FBJ osteosarcoma oncogene (C-FOS), Regulator of G-protein signaling 1 (RGS1), and FBJ osteosarcoma oncogene B (FOSB) (23). All genes could be detected by qRT-PCR, but only 2 genes were significantly up-regulated by mechanical loading, namely EGR1 and C-FOS (Figure 2, Table 3).

Many of the later genes were related to inflammation

3 hours after loading, 136 genes were regulated according to the microarray analysis Day 3 (Table 4). 41 of these genes might be involved in inflammation. 19 of the inflammation-related genes were chosen for confirmation by qRT-PCR. All genes could be detected and 7 genes were significantly up-regulated by loading, namely Chemokine (C-C motif) ligand 20 (CCL20), Chemokine (C-C motif) ligand 7 (CCL7), Interleukin-6 (IL6), Nuclear factor interleukin 3 regulated (NFIL3), Pentraxin related gene (PTX3), Suppressor of cytokine signaling 1 (SOCS1), and Toll-like receptor 2 (TLR2) (Figure 3, Table 3).

Many functions were related to inflammation

All genes regulated 3 hours after loading, according to the microarray analysis, were analyzed by Ingenuity Pathway analysis software. Overall, most of the functions affected by loading Day 3 could be connected to inflammation. Cellular movement was affected (Table 5) and most of its specific functions were connected to leukocytes, for example chemotaxis of leukocytes and leukocyte migration (Table 6). Other functions that could be connected to inflammation Day 3 were cellular growth and proliferation (generation and stimulation of lymphocytes/leukocytes), cellular development (differentiation of leukocytes) and cell-to-cell signaling and interaction (activation and binding of leukocytes). Also the predicted upstream regulators were inflammation-related Day 3, such as IL-1 β (IL1B), IFN- γ (IFNG), and TNF (Table 7).

Loading increased the number of leukocytes

Histological evaluation Day 3 showed mainly immature fibrous tissue interspersed by adipocytes and leukocytes (Figure 4). A majority of the cells in the healing tendon tissue were stained for leukocytes (CD45) and slightly fewer for macrophages (CD68). There was a significant increase of leukocytes in tendons exposed to loading compared to unloaded tendons (p 0.03). However, this difference could not be seen with macrophages (p 0.69). Overall, the leukocytes and macrophages were evenly distributed around and between the tendon stumps, but with a tendency to a higher density around the proximal stump. The leukocytes and macrophages were fewer in the adipose tissue, and also in areas with more mature fibrous tissue.

Day 14

IER3 might be up-regulated immediately after mechanical loading

15 minutes after loading, 53 genes were regulated Day 14 according to the microarray analysis (Table 8). EGR1, C-FOS, EGR2 and RGS1 were among those genes. 27 of the 53 genes might be involved in inflammation. For confirmation by qRT-PCR, 13 of the inflammation-related genes were chosen. 11 other regulated genes involved in cell signaling or gene transcription were also chosen for confirmation. All genes, except 2 (IL10 and ELMOD1), could be detected (Figure 5). However, none of the 24 genes analyzed was significantly regulated by loading, although Immediate early response 3 (IER3) was close to statistical significance (p 0.052).

Inflammation seemed not to be affected

3 hours after loading, 91 genes were regulated according to the microarray analysis Day 14 (Table 9). 26 of these genes might be involved in inflammation. 9 of the inflammation-related genes were chosen for confirmation by qRT-PCR. All genes could be detected but none of them was significantly regulated by loading (Figure 6).

All genes regulated 3 hours after loading, according to the microarray analysis, were analyzed by Ingenuity Pathway analysis software. Overall, loading affected the functions of cellular growth and proliferation, cellular development, and cell-to-cell signaling and interaction Day 14, as it did Day 3 (Table 5). However, there were no specific functions Day 14, so these functions could not be connected to inflammation or leukocytes, as it could Day 3. Although, IL-1 β was still predicted to be an activated upstream regulator Day 14 (Table 7).

Eighteen genes were regulated by mechanical loading both days

3 hours after loading, 18 genes were regulated both Day 3 and Day 14 in a similar way according to the microarray analysis (Table 10). 16 were up-regulated and 2 were down-regulated by loading.

Discussion

EGR1 and C-FOS seem important in the immediate response after mechanical loading

The primary gene response after loading involved up-regulation of the transcription factors EGR1 and C-FOS Day 3. These 2 genes have previously been shown to be up-regulated by loading at day 5 in a similar study, together with EGR2, RGS1 and FOS-B (23). Interestingly, EGR1, C-FOS, EGR2 and RGS1 were also up-regulated Day 14 according to the microarray analysis, but this was not confirmed by qRT-PCR. Still, it is unlikely that these genes would be positive by microarray at two or three different time points by random, since at each time points there were only a few positive genes out of the approximately 25 000 genes analyzed. Therefore, it is likely that these genes, especially EGR1 and C-FOS, are the first genes to respond to loading during tendon healing, regardless of healing phase.

EGR1 and C-FOS are early genes, which can be up-regulated by different types of environmental stimuli including mechanical stimulation and tissue injury (5, 11, 17, 26, 48). EGR1 can also be induced by growth factors such as transforming growth factor- β (TGF- β) (58).

EGR1 is up-regulated during tendon development and seems to be important for tendon cell differentiation, collagen type I production, and can induce expression of the tendon marker scleraxis (28, 34, 55). EGR1 is also required for optimal wound healing and strongly connected to fibrosis in different tissues as an important mediator of TGF- β induced responses (5, 58). However, expression of EGR1 in healing tendons has not been associated with fibrosis or scar formation, instead EGR1 has been shown to promote tendon repair (16, 26, 28, 47).

Additionally, EGR1 has been shown to increase during tendon healing in other studies (16, 23, 28, 47), which suggests that EGR1 is an important gene during tendon healing.

EGR1 might also play a role in inflammation, as EGR-1 null mice have an attenuated inflammatory response and leukocyte activation (58), and EGR1 is also involved in macrophage differentiation (10). Interestingly, EGR1 has been shown to regulate the expression of inflammatory mediators such as IL-6, CCL7, IL-1 β , and TNF (12, 27, 49). This suggests that there might be a link between the up-regulated EGR1 at 15 minutes and the up-regulation of IL-6 and CCL7 3 hours later Day 3. It is also possible that IL-1 β and TNF, which were predicted upstream regulators 3 hours after loading Day 3, have been activated by EGR1.

FOS proteins like C-FOS, together with Jun proteins, form a complex called activator protein-1 (AP-1). AP-1 is a key transcription factor in osteoblastic differentiation (29). Possibly, AP-1 might also be involved in tenocyte differentiation. Interestingly, the AP-1 complex has a binding site in the promotor for collagen type I, which is the predominating collagen in normal tendons (35).

In summary, EGR1 and C-FOS might be some kind of master regulators of the gene response after mechanical loading during tendon healing. They might improve tendon healing by promoting tendon cell proliferation and differentiation, stimulate collagen type I production, and regulate the inflammatory response.

Recruitment of leukocytes is increased by mechanical loading Day 3

3 genes involved in recruitment of leukocytes were significantly up-regulated 3 hours after loading Day 3, namely CCL7, CCL20, and PTX3. CCL7 can attract almost all types of leukocytes (50, 56), CCL20 mainly attracts lymphocytes and dendritic cells (14, 61), and PTX3 can dampen leukocyte recruitment by binding of Selectin-P (SELP) (42). These 3 genes can all be produced by fibroblasts and different leukocytes in response to inflammatory mediators such as IL-1 β and TNF (42, 44, 50, 61). CCL20 can also be activated by AP-1, the protein complex containing C-FOS and C-JUN. Selectin-E (SELE) and SELP were also up-regulated by loading according to the microarray analysis. They function as cell adhesion molecules on the surfaces of activated endothelial cells and play an important part in recruiting leukocytes to the site of injury (59).

According to Ingenuity pathway analysis, the late gene response Day 3 can largely be connected to an increased recruitment of leukocytes. For example attraction, cell movement, chemotaxis, migration, and mobilization of leukocytes were all activated functions. To confirm these results a histological evaluation was performed Day 3 which showed that there were more leukocytes (CD45⁺ cells) in the tendons 3 hours after loading compared to unloaded controls. Overall, our results suggest that the recruitment of leukocytes is increased by loading Day 3. However, this could not be shown in a similar study using flow cytometry (6). In that study, mechanical loading did not seem to increase the number of leukocytes in the healing tendons at day 3. Instead, it seemed to prolong M1 type of inflammation by delaying the switch to M2 type of inflammation. However, in the flow cytometry study, the rats were allowed full loading on their

healing tendons by unrestricted cage activity, which might not be comparable to this study where the healing tendons were only exposed to a single loading episode.

Macrophages and T cells might be affected by mechanical loading Day 3

Many of the genes significantly regulated by loading in the late gene response Day 3 can be expressed in both macrophages (TLR2 (24, 33), NFIL3 (40), SOCS1 (38), IL-6 (38), and SBNO2 (18)) and T cells (TLR2 (33), NFIL3 (40), SOCS1 (54), and IL6 (51)). TLR2 plays a critical role in the detection of pathogen-or damage-associated molecular patterns (PAMPs and DAMPs) to initiate host defense and regulate the inflammatory response (24). SBNO2 is a transcriptional corepressor, which seems to repress inflammatory gene expression (18). SOCS1 suppresses cytokine signaling, is a key regulator of inflammation, and might be involved in macrophage and T cell differentiation (38, 54). NFIL3 is a transcription factor that is involved in macrophage activation and T cell differentiation (40).

Histology showed that the leukocytes in the healing tendon Day 3 were predominantly macrophages. Others have shown that the most prominent immune cell type, 3 days after flexor tendon injury, was from the monocyte/macrophage lineage (41). Additionally, there are strikingly more macrophages than T cells in the healing Achilles tendons at day 3 according to flow cytometry data (6). Interestingly, M1 macrophages are efficient producers of IL-1 β , TNF and IL-6 and associated with TLR, NF κ B, and IFN- γ (38), which were all up-regulated or predicted upstream regulators in the response to loading Day 3. This suggests that the inflammation-related genes regulated by loading Day 3 might be expressed in macrophages, probably M1 macrophages.

IL-6 might regulate inflammation and tendon healing Day 3

IL-6 had the highest fold change (FC 6.9) among all genes affected by mechanical loading Day 3. It is a pro-inflammatory cytokine and is expressed by macrophages, T cells and fibroblasts (38, 51). Apart from its important role during immune responses (51), it is also important for tendon structure, function, and healing. A deficiency of IL-6 leads to thinner tendons with higher elastic modulus (37) whereas injections of IL-6 into intact human tendons stimulates collagen synthesis (1). Furthermore, IL-6 can be up-regulated by mechanical loading in intact human tendons (1, 32). This suggests that IL-6 is important for tendon structure and function and can be induced by mechanical loading. Additionally, IL-6 might also be needed for a normal healing process, as deficiency of IL-6 impairs tendon healing (36). Accordingly, IL-6 is expressed during the first week after Achilles tendon transection in rats (21). In summary, IL-6 might both regulate the inflammatory response and improve tendon healing, possibly by increasing collagen synthesis.

Inflammation seems mainly un-affected by mechanical loading Day 14

There were no inflammation-related genes confirmed by qRT-PCR 3 hours after loading Day 14. This corresponds to the results from the Ingenuity pathway analysis, showing no specific functions connected to inflammation. However, IL-1 β was predicted as an upstream regulator which suggests that there might still be some regulation of inflammation. It is possible that some of the 11 inflammation-related genes that were not chosen for confirmation by qRT-PCR, were significantly regulated.

Genes regulated both days might affect inflammation, extracellular matrix, and fibroblasts

18 genes were regulated both Day 3 and Day 14, 3 hours after loading, in a similar way according to the microarray analysis. These genes might be important for the response to loading in tendon healing, regardless of the healing phase.

Many of these 18 genes could once again be connected to inflammation but also tissue injury and healing, extracellular matrix remodeling, migration, and proliferation (Table 10). TNFRSF12A was up-regulated by loading both days. It is a cell surface receptor and thought to be a major physiologic mediator of tissue repair after acute injury by regulating inflammation, tissue remodeling, migration, differentiation, apoptosis and angiogenesis (4, 9). Interestingly, TNFRSF12A specifically promotes proliferation and increases collagen production in fibroblasts (9). ADAM metalloproteinase 4 (ADAMTS4) was also up-regulated by loading on both days. It is an extracellular matrix protease, expressed in tendons, which can cleave different proteoglycans (15). Expression of ADAMTS4 can be induced by IL-1 β and TGF- β (15, 57). Interestingly, ADAMTS4 might be important during tendon healing as it is increased in ruptured tendons compared to intact tendons in humans (30). In accordance to our results, ADAMTS4 has also been shown to be up-regulated by loading in 5 day healing tendons (21). Hyaluronan synthase 1 (HAS1), glutamine-fructose-6-phosphate aminotransferase 2 (GFPT2) and UDP-N-acetylhexosamine pyrophosphorylase (UAP1) are strongly connected to each other and were all up-regulated by loading both days. HAS1 is an enzyme responsible for one type of cellular hyaluronan synthesis. GFPT2 and UAP1 are, according to UniProt, enzymes that produce uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a substrate that is important for HAS1 activity (53). HAS1 can be induced by IL-1 β , TNF and TGF- β and may play a role during

inflammation as the hyaluronan coat produced by HAS1 mediates a leukocyte recruiting matrix and stimulate transcription of genes related to inflammation (53). Additionally, fibroblasts can express HAS1, and matrix produced by HAS1 is typical for cells of mesenchymal origin such as fibroblasts (45, 53). Syndecan-4 (SDC4) was also up-regulated by loading both days. It is a cell surface receptor which is a central mediator of cell adhesion, migration, proliferation and mechanotransduction (19). Interestingly, SDC4 has been shown to promote fibroblast migration during wound healing (8).

In summary, both during the inflammatory and the early remodeling phase of healing, mechanical loading might affect extracellular matrix remodeling, promote migration and proliferation of fibroblasts, increase collagen production, regulate the immune response, and increase leukocyte recruitment.

Limitations

We have only investigated gene expression and have not studied how this correlates with protein levels. It is unclear how changes in gene expression influence protein production, protein secretion, and cell signaling. The gene expression was studied in the whole healing tissue, which is a mixture of different cell types, so we can only speculate which cells are expressing the genes regulated by loading. Several of the regulated genes have multiple functions depending on in which cell type they are expressed, and it is therefore difficult to draw solid conclusions about their role in this context. Finally, we have not studied the gene response in normal weight bearing tendons without tendon transection which could have controlled for the effects of mechanical loading in normal rats.

Conclusion

The immediate gene response after mechanical loading in healing tendons seems to be similar, regardless of the healing phase. The transcription factors EGR1 and C-FOS might be some kind of master regulators of the gene response after mechanical loading during tendon healing. They might regulate the inflammatory response and promote tendon cell proliferation, differentiation and collagen production.

During the inflammatory phase of healing, the late gene response after mechanical loading is strongly connected to inflammation, which is not seen in the early remodeling phase. Mechanical loading seems to increase the recruitment of leukocytes and regulate the inflammatory response by affecting macrophages and T cells during the inflammatory phase of tendon healing. Finally, IL-6 is highly affected by mechanical loading and might be important for both regulating the inflammatory response and improving tendon healing.

This study shows that mechanical loading strongly influences the tissue in healing tendons. As little as a few minutes of mechanical stimulation can change the expression of hundreds of genes and also change the immune cell population. This suggests that even small amounts of mechanical loading might be important during rehabilitation of patients with tendon injuries.

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Disclosure

The authors report no conflicts of interest.

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Figure legends

Figure 1. Experimental design. All rats had their right Achilles tendon transected and were tail suspended continuously from Day 1. At the day of euthanasia (Day 3 or Day 14) the rats were either euthanized directly to serve as controls (A) or were exposed to loading by walking on a treadmill for 5 minutes. Those exposed to loading were either euthanized 15 minutes after walking had started (B) or tail suspended again and euthanized 3 hours later (C).

Figure 2. Genes regulated 15 minutes after mechanical loading in healing tendons Day 3.

C6 and FCNB were significantly regulated by loading according to the microarray analysis (Student's t-test; N=4). C-FOS and EGR1 were significantly up-regulated by loading according to qRT-PCR (Mann Whitney U-test; N=7 loading, 6 unloading). FOSB was not detected by microarray. • = fold change > 1.5 or < -1.5 together with $p < 0.05$. ▫ = fold change > 1.5 and $p = 0.07$

Figure 3. Genes regulated 3 hours after mechanical loading in healing tendons Day 3.

All of these genes were significantly regulated by loading according to the microarray analysis (Student's t-test, N=4). CCL20, CCL7, IL-6, NFIL3, PTX3, SOCS1, and TLR2 were significantly up-regulated by loading according to qRT-PCR (Mann Whitney U-test, N=4 loading, 6 unloading). RHOH, SELE and SOCS3 were not detected by qRT-PCR. • = fold change > 1.5 or < -1.5 together with $p < 0.05$. ▫ = fold change > 1.5 and $p = 0.063$

Figure 4. Histology of healing tendons Day 3. A) Unloaded healing tendon (Rat A) stained with Hematoxylin and Eosin. The old tendon stump is seen in the upper part of the picture on the

right side. B) Loaded healing tendon (Rat H) stained for CD68⁺ cells (macrophages) and detected by immunoperoxidase staining (brown) in 10x magnification. The picture corresponds to approximately the same area as seen in D. C) Unloaded healing tendon (Rat A) stained for CD45⁺ cells (leukocytes) and detected by immunoperoxidase staining (brown) in 10x magnification. D) Loaded healing tendon (Rat H) stained for CD45⁺ cells and detected by immunoperoxidase staining in 10x magnification. Picture C and D corresponds to an area with the highest amount of leukocytes in the healing tissue (brown cells). E) Histological evaluation of leukocyte (CD45) and macrophage (CD68) staining. The table shows the mean of two independent investigators. The specimens were graded from 1-3 in 3 different magnifications (4x, 10x and 20x), i.e. grading ranges from 3-9 (where 9 correspond to a high number of stained cells).

Figure 5. Genes regulated 15 minutes after mechanical loading in healing tendons Day 14.

All of these genes were significantly regulated by loading according to the microarray analysis (Student's t-test, N=4) except EGR2 and FOSB which were not detected. No genes were significantly regulated according to qRT-PCR (Mann Whitney U-test, N=6 loading, 5 unloading). ELMOD1 and IL-10 were not detected by qRT-PCR. ■ = fold change > 1.5 and p = 0.052

Figure 6. Genes regulated 3 hours after mechanical loading in healing tendons Day 14. All

of these genes were significantly regulated by loading according to the microarray analysis (Student's t-test, N=4). No genes were significantly regulated according to qRT-PCR (Mann Whitney U-test, N=5). LILRA5 and ELMOD1 were not detected by qRT-PCR.

Table 1. Primers used for quantitative real-time PCR.

Table 2. Microarray: Genes regulated 15 minutes after loading Day 3

11 genes were significantly regulated according to the microarray results (Student's t-test, N=4). 3 genes might play a role in the inflammatory response according to gene ontology (*). 2 genes were chosen for confirmation with qRT-PCR (bold).

Table 3. Fold change and confidence interval for regulated genes. Foldchange (FC) from qRT-PCR analysis. The result is analyzed with Mann-Whitney U test (N=4-7).

Table 4. Microarray: Genes regulated 3 hours after loading Day 3

136 genes were significantly regulated according to the microarray analysis (Student's t-test, N=4). 41 genes might play a role in the inflammatory response according to gene ontology (*). 19 genes were chosen for confirmation with qRT-PCR (bold).

Table 5. Top five of molecular and cellular functions in Ingenuity Pathway Analysis. All genes regulated 3 hours after loading Day 3 and Day 14 (according to the microarray analysis, fold change ≥ 1.5 or ≤ -1.5 together with a p-value of < 0.05 , Student's t-test, N=4) were analyzed by Ingenuity Pathway analysis software. "Molecules involved" means how many significantly regulated genes in the microarray analysis were involved in that specific molecular and cellular function. All specific functions had an activation z-score > 2 , as a definition for activation.

Table 6. Specific functions from Ingenuity pathway analysis, 3 hours after loading Day 3.

All genes regulated 3 hours after loading Day 3 (according to microarray analysis, fold change ≥ 1.5 or ≤ -1.5 together with a p-value of < 0.05 , Student's t-test, N=4) were analyzed by Ingenuity Pathway analysis software. All specific functions in the table had a p-value < 0.05 . A means activation z-score. B means number of genes that can be connected to this function and was significantly regulated according to the microarray analysis. C means belongs to the molecular and cellular functions of: 1) cellular movement 2) cellular growth and proliferation 3) cellular development 4) cell death and survival 5) cell-to-cell signaling and interaction.

Table 7. Top five of upstream regulators in Ingenuity Pathway Analysis. All genes regulated 3 hours after loading Day 3 and Day 14 (according to the microarray analysis, fold change ≥ 1.5 or ≤ -1.5 together with a p-value of < 0.05 , Student's t-test, N=4) were analyzed by Ingenuity Pathway analysis software.

Table 8. Microarray: Genes regulated 15 minutes after loading Day 14

53 genes were significantly regulated according to the microarray analysis (Student's t-test, N=4). 27 genes might play a role in the inflammatory response according to gene ontology (*). 22 genes were chosen for confirmation with qRT-PCR (bold), some of them might play a role in the inflammatory response (*) and some might be involved in cell signaling and gene transcription.

Table 9. Microarray: Genes regulated 3 hours after loading Day 14

91 genes were significantly regulated according to the microarray analysis (Student's t-test, N=4). 26 genes might play a role in the inflammatory response according to gene ontology (*). 9 genes were chosen for confirmation with qRT-PCR (bold).

Table 10. Genes regulated 3 hours after loading Day 3 and Day 14. Genes regulated on both days according to the microarray analysis (Student's t-test, N=4). FC means fold change. p means p-value. Biological process: I means inflammation, TI&H means tissue injury and healing, EM means extracellular matrix, M means migration, and P means proliferation.

Gene symbol	Gene name	Assay identification	Accession
Atf3	Activating transcription factor 3	Rn00563784_m1	NM_012912.2
C3	Complement component 3	Rn00566466_m1	NM_016994.2
C6	Complement component 6	Rn00598544_m1	NM_176074.3
Ccl20	Chemokine (C-C motif) ligand 20	Rn00570287_m1	NM_019233.1
Ccl3	Chemokine (C-C motif) ligand 3	Rn01464736_g1	NM_013025.2
Ccl6	Chemokine (C-C motif) ligand 6	Rn01456400_m1	NM_001004202.2
Ccl7	Chemokine (C-C motif) ligand 7	Rn01467286_m1	NM_001007612.1
Ccr5	Chemokine (C-C motif) receptor 5	Rn02132969_s1	NM_053960.3
Cd80	Cd80 molecule	Rn00709368_m1	NM_012926.1
Cxcl10	Chemokine (C-X-C motif) ligand 10	Rn01413889_g1	NM_139089.1
Cxcl13	Chemokine (C-X-C motif) ligand 13	Rn01450028_m1	NM_001017496.1
Egr1	Early growth response 1	Rn00561138_m1	NM_012551.2
Egr2	Early growth response 2	Rn00586224_m1	NM_053633.1
Egr3	Early growth response 3	Rn00567228_m1	NM_017086.1
Elmod1	ELMO/CED-12 domain containing 1	Rn01421567_m1	NM_001191579.1
Fcnb	Ficolin B	Rn00586231_m1	NM_053634.1
Fos	FBJ osteosarcoma oncogene (c-Fos)	Rn00487426_g1	NM_022197.2
Fosb	FBJ osteosarcoma oncogene B	Rn00500401_m1	NM_001256509.1
Ier2	Immediate early response 2	Rn02531674_s1	NM_001009541.1
Ier3	Immediate early response 3	Rn03993554_g1	NM_212505.2
Il10	Interleukin 10	Rn00563409_m1	NM_012854.2
Il1b	Interleukin 1 beta	Rn00580432_m1	NM_031512.2
Il1rl1	Interleukin 1 receptor-like 1	Rn01640664_m1	NM_001127689.1
Il4ra	Interleukin 4 receptor	Rn01507024_m1	NM_133380.2
Il6	Interleukin 6	Rn01410330_m1	NM_012589.2
Itga2	Integrin, alpha 2	Rn01489315_m1	XM_345156.7
Junb	Jun B proto-oncogene	Rn00572994_s1	NM_021836.2
Lira5	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	Rn04222694_mH	NM_001076793.1
Msr1	Macrophage scavenger receptor 1	Rn01488115_m1	NM_001191939.1
Nfil3	Nuclear factor, interleukin 3 regulated	Rn01434874_s1	NM_053727.2
Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Rn01474603_m1	NM_001107095.1
Ptges	Prostaglandin E synthase	Rn00572047_m1	NM_021583.3
Ptgs2	Prostaglandin-endoperoxide synthase 2 (COX-2)	Rn01483828_m1	NM_017232.3
Ptx3	Pentraxin 3, long	Rn01769850_m1	NM_001109536.1
Rgs1	Regulator of G-protein signaling 1	Rn01483325_m1	NM_019336.1
Rgs2	Regulator of G-protein signaling 2	Rn00584932_m1	NM_053453.2
Rgs6	Regulator of G-protein signaling 6	Rn01517937_m1	NM_019342.1
Rhoh	Ras homolog family member H	Rn01417510_m1	NM_001013430.1
Ripk3	Receptor-interacting serine-threonine kinase 3	Rn01481949_g1	NM_139342.1
Sbno2	Strawberry notch homolog 2 (Drosophila)	Rn01408530_m1	NM_001108068.1
Sele	Selectin E	Rn00594072_m1	NM_138879.1
Selp	Selectin P	Rn00565416_m1	NM_013114.1
Socs1	Suppressor of cytokine signaling 1	Rn00595838_s1	NM_145879.1
Tlr2	Toll-like receptor 2	Rn02133647_s1	NM_198769.2
Tnfrsf12a	Tumor necrosis factor receptor superfamily, member 12a	Rn00710373_m1	NM_181086.2
Xcr1	Chemokine (C motif) receptor 1	Rn03037149_s1	NM_001106871.1

Gene	Gene name	FC	p
C6*	complement component 6	1.64	0.016
Ccdc152	coiled-coil domain containing 152	1.50	0.027
Fcnb*	ficolin B	-1.92	0.034
Mir323	microRNA mir-323	1.63	0.040
Mir487b	microRNA mir-487b	1.55	0.015
Rnf213	ring finger protein 213	1.54	0.039
Rnf213	ring finger protein 213	1.58	0.037
Rnf213	ring finger protein 213	1.55	0.039
Rnf213	ring finger protein 213	1.57	0.049
Slc2a5	solute carrier family 2 (facilitated glucose/fructose transporter). member 5	1.55	0.013
Zbp1*	Z-DNA binding protein 1	1.96	0.050

		Confidence interval limits			p-value
	Gene	FC	Lower	Higher	
Day 3 15 min	C-FOS	4.6	2.17	11.29	< 0.05
	EGR1	2.5	1.42	4.78	< 0.05
	RGS1	1.9	0.97	3.71	0.07
Day 3 3 h	CCL20	3.6	2	6.26	< 0.05
	CCL7	4.2	1.08	7.3	< 0.05
	IL4RA	3.7	0.8	7.26	0.063
	IL6	7.2	2.2	14.59	< 0.05
	NFIL3	2.2	1.11	3.11	< 0.05
	PTX3	4.1	1.18	7.73	< 0.05
	SBNO2	2.5	0.92	4.57	0.063
	SELP	3.2	0.67	6.88	0.11
	SOCS1	2.9	1.21	4.81	< 0.05
	TLR2	3	1	6.42	< 0.05
Day 14 15 min	IER3	1.6	1	2.36	0.052

Gene	Gene name	FC	p
Adamts1*	ADAM metallopeptidase with thrombospondin type 1 motif. 1	1.84	0.004
Adamts4*	ADAM metallopeptidase with thrombospondin type 1 motif. 4	2.37	0.001
Adamts9	a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif. 9	1.81	0.008
Adora2b	adenosine A2B receptor	2.19	0.004
Agtr1a	angiotensin II receptor. type 1a	1.63	0.040
Arntl	aryl hydrocarbon receptor nuclear translocator-like	1.63	0.005
Baz1a	bromodomain adjacent to zinc finger domain. 1A	1.55	0.009
C3*	complement component 3	1.85	0.018
Ccl20*	chemokine (C-C motif) ligand 20	2.48	0.029
Ccl7*	chemokine (C-C motif) ligand 7	1.72	0.012
Ccnf	cyclin F	1.51	0.011
Ccnjl	cyclin J-like	1.79	0.010
Ccr5*	chemokine (C-C motif) receptor 5	1.67	0.011
Cd80*	Cd80 molecule	1.69	0.034
Cebpb*	CCAAT/enhancer binding protein (C/EBP). beta	1.60	0.029
Chst11	carbohydrate (chondroitin 4) sulfotransferase 11	1.64	0.001
Chsy1	chondroitin sulfate synthase 1	1.64	0.002
Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	1.52	0.018
Crem	cAMP responsive element modulator	2.02	0.007
Ctgf	connective tissue growth factor	-1.84	0.000
Cth	cystathionase (cystathionine gamma-lyase)	1.59	0.005
Cxcl10*	chemokine (C-X-C motif) ligand 10	1.69	0.015
Dbp	D site of albumin promoter (albumin D-box) binding protein	-1.57	0.001
Dusp5	dual specificity phosphatase 5	1.59	0.040
E2f5	E2F transcription factor 5	1.64	0.001
Epm2a	epilepsy. progressive myoclonus type 2A	-1.52	0.006
Ereg*	epiregulin	1.56	0.014
Fbxo32	F-box protein 32	-1.57	0.006
Fcnb*	ficolin B	3.30	0.012
Fgf23	fibroblast growth factor 23	3.03	0.001
Fmo5	flavin containing monooxygenase 5	1.89	0.002
Fosl1	fos-like antigen 1	2.64	0.026
G0s2	G0/G1switch 2	-1.51	0.007
Gadd45g	growth arrest and DNA-damage-inducible. gamma	1.55	0.004
Gbx2	gastrulation brain homeobox 2	2.05	0.000
Gch1	GTP cyclohydrolase 1	1.54	0.013
Gfpt2*	glutamine-fructose-6-phosphate transaminase 2	1.82	0.003
Gnat1	guanine nucleotide binding protein. alpha transducing 1	1.87	0.044
Gpr155	G protein-coupled receptor 155	-1.52	0.032
Gpr34*	G protein-coupled receptor 34	-1.53	0.017
Gpr39	G protein-coupled receptor 39	1.62	0.003

Gprc5a	G protein-coupled receptor. family C. group 5. member A	1.55	0.031
Has1*	hyaluronan synthase 1	3.22	0.017
Has2*	hyaluronan synthase 2	2.26	0.001
Heatr3*	HEAT repeat containing 3	1.58	0.006
Hk2	hexokinase 2	1.82	0.017
Hs3st3b1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	1.54	0.002
Hspb8	heat shock protein B8	1.52	0.018
Hsph1	heat shock 105kDa/110kDa protein 1	1.67	0.007
Il28ra*	interleukin 28 receptor alpha	1.56	0.005
Il4ra*	interleukin 4 receptor. alpha	1.67	0.004
Il6*	interleukin 6	3.78	0.011
Inhba	inhibin beta-A	3.84	0.021
Irf7*	interferon regulatory factor 7	1.62	0.019
Isg15*	ISG15 ubiquitin-like modifier potassium voltage-gated channel. subfamily H (eag-related). member 1	1.63	0.003
Kcnh1		1.56	0.023
Klf11	Kruppel-like factor 11	-1.64	0.010
LOC500300	similar to hypothetical protein MGC6835	-1.57	0.016
LOC686567	similar to Epiplakin	-1.54	0.017
Ly6c	Ly6-C antigen	1.59	0.048
Mcoln2	mucolipin 2	1.60	0.027
Mcoln3	mucolipin 3	1.76	0.011
Mfap3l	microfibrillar-associated protein 3-like	1.99	0.005
Mir323	microRNA mir-323	2.12	0.012
Mir539	microRNA mir-539	1.54	0.019
Mx1*	myxovirus (influenza virus) resistance 1	1.98	0.044
Myc	myelocytomatosis oncogene	1.56	0.017
Ncapg2	non-SMC condensin II complex. subunit G2	1.57	0.001
Nfil3*	nuclear factor. interleukin 3 regulated	1.64	0.003
Nfya	nuclear transcription factor-Y alpha	1.56	0.009
Ngf	nerve growth factor (beta polypeptide)	1.50	0.001
Npas2	neuronal PAS domain protein 2	1.52	0.048
Nr1d1*	nuclear receptor subfamily 1. group D. member 1	-2.82	0.000
Nrg1	neuregulin 1	2.02	0.042
Nrk	Nik related kinase	1.69	0.043
Oas1a*	2'-5' oligoadenylate synthetase 1A	1.76	0.039
Oasl2	2'-5' oligoadenylate synthetase-like 2	1.84	0.016
Orc1l	origin recognition complex. subunit 1-like (yeast)	1.52	0.013
Pappa	pregnancy-associated plasma protein A	1.89	0.014
Pck1	phosphoenolpyruvate carboxykinase 1 (soluble)	-1.89	0.000
Pdk4	pyruvate dehydrogenase kinase. isozyme 4	-1.52	0.033
Peg12	paternally expressed 12	1.59	0.001
Penk	proenkephalin	1.74	0.012

Per1	period homolog 1 (Drosophila)	-1.64	0.042
Per3	period homolog 3 (Drosophila)	-2.11	0.000
Pik3ip1	phosphoinositide-3-kinase interacting protein 1	-2.20	0.005
Plac8	placenta-specific 8	1.50	0.000
Plau	plasminogen activator. urokinase	1.51	0.014
Plaur	plasminogen activator. urokinase receptor	1.51	0.041
Plek	pleckstrin	1.52	0.037
Polr1b	polymerase (RNA) I polypeptide B	1.62	0.014
Pot1b	protection of telomeres 1B	-1.54	0.001
Pragmin	pragma of Rnd2	2.03	0.025
Ptges*	prostaglandin E synthase	1.56	0.027
Ptx3*	pentraxin related gene	2.66	0.004
Rab20	RAB20. member RAS oncogene family	1.51	0.029
RGD1560289	similar to chromosome 3 open reading frame 20	-1.57	0.005
RGD1562846	similar to Docking protein 5 (Downstream of tyrosine kinase 5) (Protein dok-5)	2.25	0.041
RGD1563073	similar to SIGLEC-like 1	1.69	0.005
RGD1563982	similar to F-box only protein 27	1.76	0.012
RGD1565316	similar to sphingomyelin phosphodiesterase 3. neutral membrane	1.55	0.046
Rhbdf2*	rhomboid 5 homolog 2 (Drosophila)	1.70	0.008
Rhoh*	ras homolog gene family. member H	1.52	0.016
Ripk3*	receptor-interacting serine-threonine kinase 3	1.69	0.009
Rnd1	Rho family GTPase 1	2.28	0.007
Rpp38	ribonuclease P/MRP 38 subunit (human)	1.57	0.001
Sdc4*	syndecan 4	1.85	0.028
Sele*	selectin E	1.67	0.008
Selp*	selectin P	1.99	0.008
Serpine1	serine (or cysteine) peptidase inhibitor. clade E. member 1	1.58	0.032
Slc39a14	solute carrier family 39 (zinc transporter). member 14	1.60	0.002
Slfn3	schlafen 3	1.65	0.046
Slitrk6	SLIT and NTRK-like family. member 6	-1.53	0.038
Snai1	snail homolog 1 (Drosophila)	1.52	0.006
Sned1	sushi. nidogen and EGF-like domains 1	1.68	0.010
Socs1*	suppressor of cytokine signaling 1	1.73	0.027
Socs3*	suppressor of cytokine signaling 3	1.78	0.011
Star	steroidogenic acute regulatory protein	-1.67	0.041
Stx11 *	syntaxin 11	1.54	0.019
Syt15	synaptotagmin XV	-1.51	0.014
Tef	thyrotrophic embryonic factor	-1.70	0.001
Tfpi2	tissue factor pathway inhibitor 2	2.26	0.001
Tlr2*	toll-like receptor 2	1.69	0.011
Tmem2	transmembrane protein 2	1.78	0.005
Tnfrsf12a*	tumor necrosis factor receptor superfamily. member 12a	1.52	0.007

Trib1	tribbles homolog 1 (Drosophila)	1.56	0.040
Trim30	tripartite motif-containing 30	1.51	0.004
Trmt61a	tRNA methyltransferase 61 homolog A (S. cerevisiae)	1.53	0.011
Tsc22d3*	TSC22 domain family. member 3	-1.59	0.005
Tubb3	tubulin. beta 3	1.59	0.030
Uap1*	UDP-N-acetylglucosamine pyrophosphorylase 1	2.05	0.003
Uap1*	UDP-N-acetylglucosamine pyrophosphorylase 1	2.00	0.001
Urb2	URB2 ribosome biogenesis 2 homolog (S. cerevisiae)	1.50	0.012
Wdr89	WD repeat domain 89	1.51	0.023
Vegfa	vascular endothelial growth factor A	1.68	0.017
Zbp1*	Z-DNA binding protein 1	1.79	0.032
Zfp185	zinc finger protein 185	1.57	0.015

	Molecular and cellular functions	p-value	Molecules involved	Specific functions
Day 3	Cellular movement	< 0.001	47	34
	Cellular growth	< 0.001	71	10
	Cellular development	< 0.001	69	7
	Cell death and survival	< 0.001	69	4
	Cell-to-cell signaling and interaction	< 0.001	43	26
Day 14	Cellular growth and proliferation	< 0.05	37	0
	Cellular development	< 0.05	29	0
	Cell-to-cell signaling and interaction	< 0.05	22	0
	Carbohydrate metabolism	< 0.05	11	0
	DNA replication, recombination, and repair	< 0.05	12	0

Specific function	A	B	C	Specific function	A	B	C
Activation of:				Generation of:			
antigen presenting cells	2.9	11	5	cells	2.3	10	2
blood cells	3.8	23	5	lymphocytes	2.1	8	2
cells	3.8	31	5	Invasion of:			
central nervous system cells	2.2	5	5	cells	3.4	21	1
dendritic cells	2.1	6	5	tumor cell lines	3.0	14	1
leukocytes	3.6	19	5	Migration of:			
macrophages	2.4	7	5	antigen presenting cells	2.3	11	1
myeloid cells	2.8	9	5	cells	3.8	44	1
phagocytes	3.4	15	5	mononuclear leukocytes	2.2	17	1
Adhesion of:				phagocytes	2.6	16	1
immune cells	2.9	15	5	smooth muscle cells	2.1	10	1
lymphocytes	2.0	6	5	tumor cell lines	3.8	19	1
mononuclear leukocytes	2.5	8	5	Mobilization of:			
vascular endothelial cells	2.2	5	5	blood cells	2.2	6	1
Attraction of:				leukocytes	2.0	4	1
cells	2.1	6	1, 5	Proliferation of:			
leukocytes	2.1	5	1, 5	breast cell lines	2.4	6	2, 3
Binding of:				cells	2.0	69	2
blood cells	2.3	9	5	connective tissue cells	2.9	18	2
cells	2.4	22	5	ovarian cancer cell lines	2.4	6	2, 3
leukocytes	2.1	8	5	Recruitment of:			
Cell movement of:				blood cells	2.5	15	1, 5
granulocytes	3.5	47	1	granulocytes	2.4	11	1, 5
leukocytes	2.1	15	1	mononuclear leukocytes	2.2	6	1, 5
lymphocytes	2.5	25	1	myeloid cells	2.6	12	1, 5
mast cells	2.4	14	1	Stimulation of:			
mononuclear leukocytes	2.4	7	1	cells	3.3	14	2, 5
myeloid cells	2.0	22	1	leukocytes	2.6	7	2, 5
phagocytes	2.0	22	1	Others:			
smooth muscle cells	2.2	23	1	Apoptosis of leukemia cell lines	2.2	10	4
tumor cell lines	2.2	11	1	Cell survival	2.7	33	4
Chemotaxis of:				Cell viability	2.5	31	4
antigen presenting cells	3.6	23	1	Viability of tumor cell lines	2.9	17	4
cells	2.2	9	1	Expansion of cells	2.2	11	2
leukocytes	3.3	23	1	Formation of cells	3.2	16	2
myeloid cells	2.7	18	1	Interaction of leukocytes	2.4	6	5
phagocytes	2.3	16	1	Leukocyte migration	3.1	29	1
Differentiation of:				Localization of cells	2.0	6	1
blood cells	2.9	17	1	Lymphocyte migration	2.2	13	1
cells	2.6	22	3	Phagocytosis of cells	2.1	12	5
connective tissue cells	3.1	43	3				
leukocytes	3.0	17	3				
osteoclasts	2.6	20	3				
	2.6	11	3				

	Upstream regulators	p-value	Predicted activation
Day 3	IL1B	< 0.001	Activated
	IFNG	< 0.001	Activated
	TNF	< 0.001	Activated
	lipopolysaccharide	< 0.001	Activated
	NFkB (complex)	< 0.001	Activated
Day 14	IL1B	< 0.001	Activated
	TLR9	< 0.001	
	U0126	< 0.001	
	IFNAR1	< 0.001	
	poly rI:C-RNA	< 0.001	Activated

Gene	Gene name	FC	p
A2m*	alpha-2-macroglobulin	-1.73	0.004
Adfp	adipose differentiation related protein	-1.51	0.021
Apold1	apolipoprotein L domain containing 1	1.59	0.001
Atf3*	activating transcription factor 3	1.65	0.001
Btg2	BTG family. member 2	2.30	0.000
Ccl7*	chemokine (C-C motif) ligand 7	1.52	0.034
Chodl	chondrolectin	1.77	0.037
Cxcl13*	chemokine (C-X-C motif) ligand 13	-1.63	0.044
Dkk2	dickkopf homolog 2 (Xenopus laevis)	1.54	0.013
Dusp1	dual specificity phosphatase 1	1.56	0.001
Egr1	early growth response 1	1.63	0.014
Egr3*	early growth response 3	1.78	0.018
Elmod1*	ELMO/CED-12 domain containing 1	1.64	0.017
Emilin3	elastin microfibril interfacer 3	1.53	0.002
Fos	FBJ osteosarcoma oncogene	2.24	0.033
Gadd45g*	growth arrest and DNA-damage-inducible. gamma	1.74	0.000
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	-2.23	0.025
Hrasls	HRAS-like suppressor	1.52	0.019
Ier2	immediate early response 2	2.20	0.000
Ier3*	immediate early response 3	1.66	0.009
Il10*	interleukin 10	1.64	0.020
Il1b*	interleukin 1 beta	2.18	0.001
Il1rl1*	interleukin 1 receptor-like 1	-1.75	0.003
Il6*	interleukin 6	2.20	0.038
Itga2*	integrin. alpha 2	1.53	0.047
Junb*	jun B proto-oncogene	1.63	0.002
Kcnma1	potassium large conductance calcium-activated channel. subfamily M. alpha member 1	1.56	0.002
Kera	keratocan	1.79	0.027
Kif26b	kinesin family member 26B	-1.64	0.017
LOC690521	similar to High mobility group protein 1 (HMG-1) (High mobility group protein B1)	-1.57	0.020
Mir24-2	microRNA mir-24-2	1.50	0.036
Mir27a	microRNA mir-27a	1.82	0.012
Msr1*	macrophage scavenger receptor 1	-1.65	0.001
Mx2*	myxovirus (influenza virus) resistance 2	3.07	0.003
ND6	NADH dehydrogenase subunit 6	-1.64	0.024
Nfkbiz*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor. zeta	1.56	0.000
Nr4a1*	nuclear receptor subfamily 4. group A. member 1	3.81	0.005
Nr4a2*	nuclear receptor subfamily 4. group A. member 2	2.61	0.001
Nr4a3*	nuclear receptor subfamily 4. group A. member 3	3.54	0.003
Olr1058	olfactory receptor 1058	1.86	0.005
Oxtr	oxytocin receptor	-1.64	0.019
Prox1*	prospero homeobox 1	1.73	0.004

Ptgs2*	prostaglandin-endoperoxide synthase 2 (COX-2)	1.85	0.011
Retnlg	resistin-like gamma	-1.56	0.010
Rgs1*	regulator of G-protein signaling 1	1.60	0.017
Rgs2*	regulator of G-protein signaling 2	1.69	0.000
Rgs6	regulator of G-protein signaling 6	1.53	0.001
Robo2	roundabout. axon guidance receptor. homolog 2 (Drosophila)	1.71	0.048
RT1-CE2*	RT1 class I. locus CE2	-1.52	0.046
RT1-CE5*	RT1 class I. locus CE5	-1.58	0.045
Scnn1b	sodium channel. nonvoltage-gated 1. beta	1.54	0.026
Snrpd1	small nuclear ribonucleoprotein D1	2.02	0.011
Xcr1*	chemokine (C motif) receptor 1	1.64	0.003

Gene	Gene name	FC	p
Actg2	actin. gamma 2. smooth muscle. enteric	-1.71	0.023
Adamts4*	ADAM metalloproteinase with thrombospondin type 1 motif. 4	1.60	0.006
Arntl	aryl hydrocarbon receptor nuclear translocator-like	1.58	0.005
Bmp5	bone morphogenetic protein 5	-1.66	0.006
C6*	complement component 6	-1.83	0.005
Ccl3*	chemokine (C-C motif) ligand 3	-1.66	0.022
Ccl6*	chemokine (C-C motif) ligand 6	-1.63	0.041
Cd180*	CD180 molecule	-1.62	0.026
Cebpb*	CCAAT/enhancer binding protein (C/EBP). beta	1.52	0.003
Clk1	CDC-like kinase 1	1.58	0.010
Clk4	CDC like kinase 4	1.51	0.002
Crabp2	cellular retinoic acid binding protein 2	1.94	0.019
Cyp26b1	cytochrome P450. family 26. subfamily b. polypeptide 1	1.78	0.004
Dact1	dapper. antagonist of beta-catenin. homolog 1 (Xenopus laevis)	-1.64	0.000
Dio3	deiodinase. iodothyronine. type III	1.67	0.017
Elmod1*	ELMO/CED-12 domain containing 1	1.71	0.007
Epha7	Eph receptor A7	-1.66	0.000
Fkbp5*	FK506 binding protein 5	1.98	0.005
Freq	frequenin homolog (Drosophila)	1.65	0.003
Fzd8*	frizzled homolog 8 (Drosophila)	-1.58	0.007
Gabre	gamma-aminobutyric acid (GABA) A receptor. epsilon	1.67	0.023
Gata6	GATA binding protein 6	-1.69	0.003
Gbp5	guanylate binding protein 5	-1.50	0.004
Gfpt2*	glutamine-fructose-6-phosphate transaminase 2	1.62	0.042
Gnat1	guanine nucleotide binding protein. alpha transducing 1	2.02	0.008
Gpr18	G protein-coupled receptor 18	-1.57	0.001
Gpr34*	G protein-coupled receptor 34	-1.83	0.002
Has1*	hyaluronan synthase 1	2.18	0.026
Hbegf*	heparin-binding EGF-like growth factor	1.74	0.039
Heatr3*	HEAT repeat containing 3	1.52	0.014
Hoxb6	homeo box B6	1.50	0.029
Hsph1	heat shock 105kDa/110kDa protein 1	1.68	0.003
Ifit3*	interferon-induced protein with tetratricopeptide repeats 3	-1.67	0.001

Il1b*	interleukin 1 beta	1.63	0.012
Kbtbd8	kelch repeat and BTB (POZ) domain containing 8	1.50	0.009
Kif26b	kinesin family member 26B	-1.54	0.049
Lilra5*	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	-2.24	0.030
Lins	lines homolog (Drosophila)	1.51	0.017
LOC100365768	rCG43589-like	1.62	0.032
LOC686042	hypothetical protein LOC686042	1.59	0.046
LOC686781	similar to NFkB interacting protein 1	1.51	0.001
LOC690521	similar to High mobility group protein 1 (HMG-1) (High mobility group protein B1)	-1.96	0.001
Lonrf2	LON peptidase N-terminal domain and ring finger 2	1.51	0.016
Mex3b	mex3 homolog B (C. elegans)	-1.71	0.001
MGC108823	similar to interferon-inducible GTPase	-1.57	0.002
Mir134	microRNA mir-134	1.50	0.043
Mir154	microRNA mir-154	1.67	0.027
Mir22	microRNA mir-22	-1.55	0.009
Mir300	microRNA mir-300	1.82	0.031
Mir323	microRNA mir-323	1.65	0.046
Mir369	microRNA mir-369	1.53	0.046
Mir380	microRNA mir-380	1.54	0.048
Mir382	microRNA mir-382	1.77	0.039
Mir410	microRNA mir-410	2.22	0.002
Mir411	microRNA mir-411	1.60	0.011
Mir485	microRNA mir-485	1.67	0.047
Mir495	microRNA mir-495	1.67	0.013
Nedd9	neural precursor cell expressed, developmentally down-regulated 9	-1.59	0.003
Nr1d1*	nuclear receptor subfamily 1, group D, member 1	-1.99	0.000
Nr4a3	nuclear receptor subfamily 4, group A, member 3	3.06	0.027
Oxtr	oxytocin receptor	-2.16	0.011
P2rx1	purinergic receptor P2X, ligand-gated ion channel, 1	-1.55	0.025
Phlda1	pleckstrin homology-like domain, family A, member 1	1.60	0.000
Phtf2	putative homeodomain transcription factor 2	1.56	0.005
Pik3ip1	phosphoinositide-3-kinase interacting protein 1	-1.61	0.001
Pop1	processing of precursor 1, ribonuclease P/MRP subunit (S. cerevisiae)	1.52	0.010
Ppan	peter pan homolog (Drosophila)	1.51	0.002
Pprc1	peroxisome proliferator-activated receptor gamma, coactivator-related 1	1.58	0.001

PVR*	poliovirus receptor	1.52	0.001
Rasgrf1	RAS protein-specific guanine nucleotide-releasing factor 1	1.60	0.001
Retnlg	resistin-like gamma	-1.55	0.003
RGD1305184	similar to CDNA sequence BC023105	-1.81	0.000
RGD1563365	similar to DNA segment. Chr 10. Wayne State University 102. expressed	1.56	0.009
RGD1564171	RGD1564171	1.60	0.043
Ripk3*	receptor-interacting serine-threonine kinase 3	1.71	0.005
Rtp4	receptor (chemosensory) transporter protein 4	-1.70	0.035
Sbno2*	strawberry notch homolog 2 (Drosophila)	1.50	0.018
Sdc4*	syndecan 4	1.52	0.005
Siglec10*	sialic acid binding Ig-like lectin 10	-1.58	0.008
Slitrk6	SLIT and NTRK-like family. member 6	-1.67	0.014
Snai1	snail homolog 1 (Drosophila)	1.62	0.013
Snrpd1	small nuclear ribonucleoprotein D1	1.77	0.026
Socs1*	suppressor of cytokine signaling 1	1.55	0.021
Svs5	seminal vesicle secretory protein 5	1.51	0.011
Tfpi2	tissue factor pathway inhibitor 2	2.10	0.043
Trim46	tripartite motif-containing 46	1.58	0.044
Uap1*	UDP-N-acetylglucosamine pyrophosphorylase 1	1.57	0.010
Uap1*	UDP-N-acetylglucosamine pyrophosphorylase 1	1.51	0.021
Usp18*	ubiquitin specific peptidase 18	-1.53	0.010
Zbtb11	zinc finger and BTB domain containing 11	1.67	0.010
Zfp458	zinc finger protein 458	1.56	0.009

Gene	Gene name	Day 3		Day 14		Biological process
		FC	p	FC	p	
ADAMTS4	ADAM metalloproteinase 4	2.3	< 0.001	1.6	< 0.01	I, TI&H, EM, M
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1.6	< 0.05	1.5	< 0.01	I
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	1.8	< 0.01	1.6	< 0.05	I, TI&H, EM, M
GNAT1	Guanine nucleotide binding protein, alpha transducing 1	1.9	< 0.05	2.0	< 0.01	
GPR34	G protein-coupled receptor 34	-1.5	< 0.05	-1.8	< 0.01	I, M
HAS1	Hyaluronan synthase 1	3.2	< 0.05	2.1	< 0.05	I, TI&H, EM, M
HEATR3	HEAT repeat containing 3	1.6	< 0.01	1.5	< 0.05	I, TI&H
HSPH1	Heat shock 105kDa/110 kDa protein 1	1.7	< 0.01	1.7	< 0.01	TI&H
MIR323	MicroRNA mir-323	2.1	< 0.05	1.7	< 0.05	
NR1D1	Nuclear receptor subfamily 1, group D, member 1	-2.8	< 0.001	-2.0	< 0.001	I
RIPK3	Receptor-interacting serine-threonine kinase 3	1.6	< 0.01	1.7	< 0.001	I
SBNO2	Strawberry notch homolog 2	1.5	< 0.01	1.5	< 0.05	I
SDC4	Syndecan 4	1.8	< 0.05	1.5	< 0.01	I, TI&H, M, P
SNAI1	Snail homolog 1 (Drosophila)	1.5	< 0.01	1.6	< 0.05	P
SOCS1	Suppressor of cytokine signaling 1	1.7	< 0.05	1.5	< 0.05	I
TFPI2	Tissue factor pathway inhibitor 2	2.2	< 0.01	2.1	< 0.05	EM
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12a	1.5	< 0.01	1.5	< 0.01	I, TI&H, EM, M, P
UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	2.1	< 0.01	1.6	< 0.01	I, TI&H, EM, M

A) Unloaded tendon (H&E)

B) Loaded tendon (CD68)

C) Unloaded tendon (CD45)

D) Loaded tendon (CD45)

E

	ID	CD45	CD68
Unloading	A	3	4
	B	4.5	8.5
	C	5	9
	D	7	7.5
Loading	E	7	3.5
	F	8	8.5
	G	8	4
	H	9	9