

Increased mast cell degranulation and co-localization of mast cells with the NMDA receptor-1 during healing after Achilles tendon rupture

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Abstract The role of inflammation and the mechanism of tendon healing after rupture has historically been a matter of controversy. The purpose of the present study is to investigate the role of mast cells and their relation to the NMDA receptor-1 (a glutamate receptor) during healing after Achilles tendon rupture. Eight female Sprague Dawley rats had their right Achilles tendon transected. Three weeks after rupture, histological quantification of mast cell numbers and their state of degranulation was assessed by histochemistry. Co-localization of mast cell tryptase (a mast cell marker) and NMDA receptor-1 was determined by immunofluorescence. The intact left Achilles tendon was used as control. An increased number of mast cells and a higher proportion of degranulated mast cells were found in the healing Achilles tendon compared

to the intact. In addition, increased co-localization of mast cell tryptase and NMDA receptor-1 was seen in the areas of myotendinous junction, mid-tendon proper and bone tendon junction of the healing versus the intact tendon. These findings introduce a possible role for mast cells in the healing phase after Achilles tendon rupture.

Keywords Achilles tendon healing · NMDA · Mast cells · Tryptase · Rats

Introduction

The physiological mechanisms of tendon healing and pain signaling are still far from understood and the debate continues regarding the role of inflammation in healing and tendinopathy development (Abate et al. 2009; Millar et al. 2017). Patients afflicted by acute Achilles tendon rupture (ATR) also exhibit variable and long-term impaired outcomes such as pain and fatigue (Alim et al. 2016).

In peripheral tissues, the peripheral nerve system (PNS) exhibits a key role in regulating inflammation, pain signaling and healing of the damaged tissue via afferent to efferent pathways (Chiu et al. 2012). Peripheral nerve endings at the site of injury can emit potent neuromediators with the ability to modify the function of fibroblast-like cells in the healing tendon tissue (Ackermann et al. 2014; Murphy and Hart 1993). Emitted neuronal substances may also interact with resident mast cells (MCs) and macrophages to affect their function (Chiu et al. 2012; Murphy and Hart 1993). Conversely, MCs contain substances that, when emitted, may alter the function of both the PNS and tissue cells (Hart et al. 1998). It has been hypothesized that MCs residing near nerve endings may degranulate and affect the

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function of the PNS, which makes them a potential target for modulating inflammation and pain.

Glutamate is the primary excitatory mediator of the nervous system and has, via its preferred receptor, NMDAR1, been implicated in regulating tendon healing (Ackermann et al. 2014; Molloy et al. 2006b) and various pain conditions (Freemont et al. 1997) including tendinopathy (Ackermann et al. 2014). In tendon healing as well as in tendinopathic patients, a 10-fold up-regulation of NMDAR1 expression has been established (Greve et al. 2012; Molloy et al. 2006a). In tendinopathy, NMDAR1 has been observed in morphologically transformed tenocytes in the endothelial and adventitial layers of neovessel walls and in presumed sprouting nerve fibers (Murphy and Hart 1993). The nerve ingrowth in combination with the downstream effect of NMDAR1 activation may represent an important physiological regulation mechanism operative in tendon healing, pain regulation and maybe also in tendinopathy. We hypothesize that there may exist a link between NMDAR1 and MC activation in tendon healing. The aim of this study is therefore to investigate the possible role of MCs in tendon healing. To this end, we used a rat model of ATR and investigated the presence and state of activation of MCs during the healing process. Moreover, the potential communication between MCs and nerve cells was addressed by assessing the spatial relation between MCs and NMDAR1.

Materials and methods

Study design

An animal model for ATR was used to quantify and evaluate the localization of the target biomarkers in the healing Achilles tendon (AT) in comparison with the intact AT in the contralateral leg (Eliasson et al. 2009).

Animal model

Eight female Sprague Dawley rats (Taconic, Ejby, Denmark) were used (13–15 weeks old). The mean weight was 217 g. The animals were housed 2 or 3 per cage with 12:12-h light:dark cycle and given food and water ad libitum. The study was approved by the Linköping Animal Ethics Committee (Ethical number 15-15) for animal experiments and adhered to the institutional guidelines and protocols for the care and treatment of laboratory animals (Eliasson et al. 2009).

Tendon surgery and rupture

The rats were anesthetized with isoflurane gas (Forene; Abbot Scandinavia, Solna, Sweden) and given antibiotics

preoperatively (25 mg/kg; Oxytetracycline, Engemycin; Intervet, Boxmeer, The Netherlands). Analgesics were given subcutaneously pre- and postoperatively (0.045 mg/kg; Buprenorphine, Temgesic; Schering-Plough, Brussels, Belgium). The skin on the right lower leg was shaved and cleaned with chlorhexidine ethanol and surgery was performed under aseptic conditions. The tendon complex was exposed through a transverse skin incision lateral to the Achilles tendon. The plantaris tendon was removed and the AT was fully ruptured at the mid-portion of the tendon. The wound was closed with two stitches and the rats were allowed unrestricted activity during healing.

Three weeks after tendon rupture, the rats were euthanized by CO₂. The healing tendons were dissected free from extraneous soft tissue and harvested together with parts of the gastrocnemius and soleus muscle complex. The completely dissected tendon and muscle complex was quickly rinsed in saline, snap-frozen in liquid nitrogen and stored at –80°C until tissue slicing. The tendon tissue was sectioned about 12–15 µm in size and analyzed by immunohistochemistry and immunofluorescence. The non-ruptured left Achilles tendon was used as control. The plantaris tendon was removed and the Achilles tendon tissue harvested in a similar procedure.

Quantification of mast cell numbers and degranulation

To assess the numbers of MCs and the degree of degranulation, a semi-quantitative analysis was performed. Tissue samples were stained with toluidine blue solution, 0.1% toluidine blue (Sridharan and Shankar 2012) in 0.17 mM NaCl (pH = 2) as previously described (Melo et al. 2015; Sullivan-Brown et al. 2011). Three different regions of interest were marked on the tendon during counting and the total number of MCs and the proportion of degranulated MCs were counted in each field of view using original magnification of ×200. MCs were further divided into cells that were either intact (non-degranulated), partially degranulated, or extensively degranulated (Abd-El-Aleem et al. 2005; Li et al. 2012). All photos were captured using a NikonU brightfield microscope at original magnifications of ×100, ×200 or ×400.

Histochemistry and immunofluorescence

For general tissue morphology, a standard histochemistry (hematoxylin and eosin, H&E staining) protocol was followed to evaluate the basic histological differences between the intact and healing AT (Ahmed et al. 2012; Christensen et al. 2015). The expression and localization of NMDAR1 and MC tryptase was visualized by the immunofluorescence technique. For this, fresh-frozen tissue samples were initially allowed to thaw for 10 min at room

temperature. Tissue samples were then washed with phosphate-buffered saline (PBS) for 5 min and immediately fixed with cold acetone (from -20°C) followed by immunofluorescence as described (Ackermann et al. 2003). For the NMDAR1 staining, sections were first blocked with normal horse serum, followed by rinsing with PBS ($3\times$) and incubated overnight (room temperature, in the dark) with primary antibody: monoclonal mouse anti-NMDAR1 (1:100 in PBS-BSA 0.1%, Triton 0.3%; BD Biosciences, Stockholm, Sweden). For tryptase staining, sections were blocked with either normal horse serum (for single tryptase staining) or normal goat serum (for double tryptase/NMDAR1 staining), followed by washing with PBS ($3\times$ 5 min). After the first blocking step, sections were blocked to quench non-specific binding of avidin (see final step) using an avidin-biotin blocking kit (Vector Laboratories). After washing, sections were incubated with primary antibody overnight (room temperature, in the dark and in wet conditions). Single staining for tryptase was performed using a mouse monoclonal anti-tryptase antibody (1:2000 dilution; MAB 1222; CHEMICON[®] International). For the double-staining experiments, a rabbit anti-tryptase antiserum was used (1:500 dilution). Next, the tissue sections were washed in PBS ($3\times$ 5 min) and then incubated for 60 min (on a shaker) with biotinylated secondary antibody. As secondary

antibody, biotinylated horse anti-mouse Ig (1:250 dilution in PBS-BSA 0.1%) was used for NMDAR1 staining. For detection of the rabbit anti-tryptase antibody, biotinylated goat-anti rabbit Ig (1:250 dilution in PBS-BSA 0.1%) was used as secondary antibody. Finally, sections were incubated with streptavidin-Cy2 (for tryptase; 1:2,000; Amersham, Poole, UK) or streptavidin-Cy3 (for NMDAR1; 1:5,000; Amersham). For the double-staining, tryptase and NMDAR1 staining was performed consecutively to prevent non-specific signals (Christensen et al. 2015). For visualization of nuclei, DAPI (4,6-diamidino-2-phenylindole, Invitrogen) staining was performed. Digital images were captured using a Nikon fluorescence microscope (Nikon Eclipse 90i; Japan) equipped with a CCD camera (DS-Qi1 Monochromatic Digital Camera). All photographs were taken at original magnifications of $\times 200$ or $\times 400$.

Statistical analysis

Data analyses to compare number of mast cells in each region or to compare degranulation state between intact and healing tendons were performed by non-parametric Mann–Whitney *U* test using the GraphPad Prism software (v.7.02). A repeated measures 2-way ANOVA with granulation state and region as the two variables was used to analyze if there

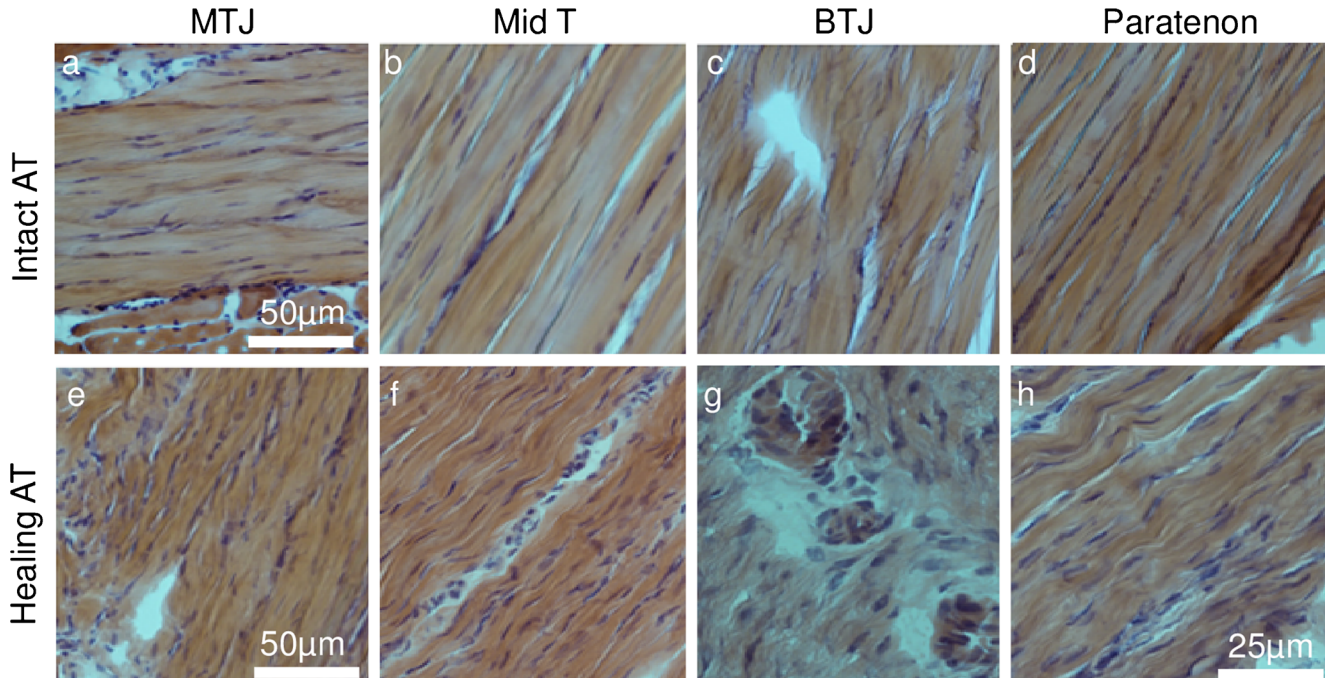


Fig. 1 Basic histological differences in intact and healing Achilles tendon. Haematoxylin and eosin staining of longitudinal sections of intact and healing rat Achilles tendon. The intact Achilles tendon (**a–d**) exhibits well-organized collagen fibers with a sparse amount of tendon cells, tightly packed between the collagen bundles in four different regions of interest of the tendon: muscle–tendon junction (**a**), mid-tendon

(**b**), bone–tendon junction (**c**) and the paratenon (**d**). In healing tendon (**e–h**), the structure is disorganized, the collagen becomes wavy, tenocytes change their morphology, the number of cells is increased and vascular ingrowth is seen. *Scale bar* (**b–d**, **f–h**) 25 μm , (**a**, **e**) 50 μm . *MTJ* muscle–tendon junction, *Mid T* mid-tendon, *BTJ* bone–tendon junction, *AT* Achilles tendon

was a difference in each factor or if there was an interaction between these factors. Intact and healing tendons were analyzed separately. P values <0.05 were considered significant.

Results

Healing tendons exhibit increased cellularity and vascularity

Intact tendons exhibited normal tissue morphology in the tendon proper, paratenon and other regions of the tendon, i.e., a normal and organized collagen structure was seen, tenocytes were tightly packed between the collagen bundles and no hyper-cellularity or evidence of vascular changes were found in the intact tissue (Fig. 1a–d). Conversely, tissue sections from ruptured AT displayed

classical signs of tendon healing, including aberrant cell morphology, increased cellularity, disorganized tissue structure and signs of newly formed blood vessels with vascular ingrowth into the tendon tissue (Fig. 1e–h). These features were apparent in all of the different regions of interest in the tendon after rupture, i.e., muscle–tendon junction (MTJ), mid-tendon, bone–tendon junction (BTJ) and the paratenon.

Increased numbers of degranulated MCs in healing compared to intact AT

To assess the presence of MCs and their extent of activation, AT tissues were stained with toluidine blue (Fig. 2a). In control, intact tendon, MCs were visible in MTJ, mid-tendon, BTJ and in the paratenon (Fig. 2b; left panels). Moreover, it was observed that a majority of the MCs appeared intact (non-degranulated), i.e., did not show signs of

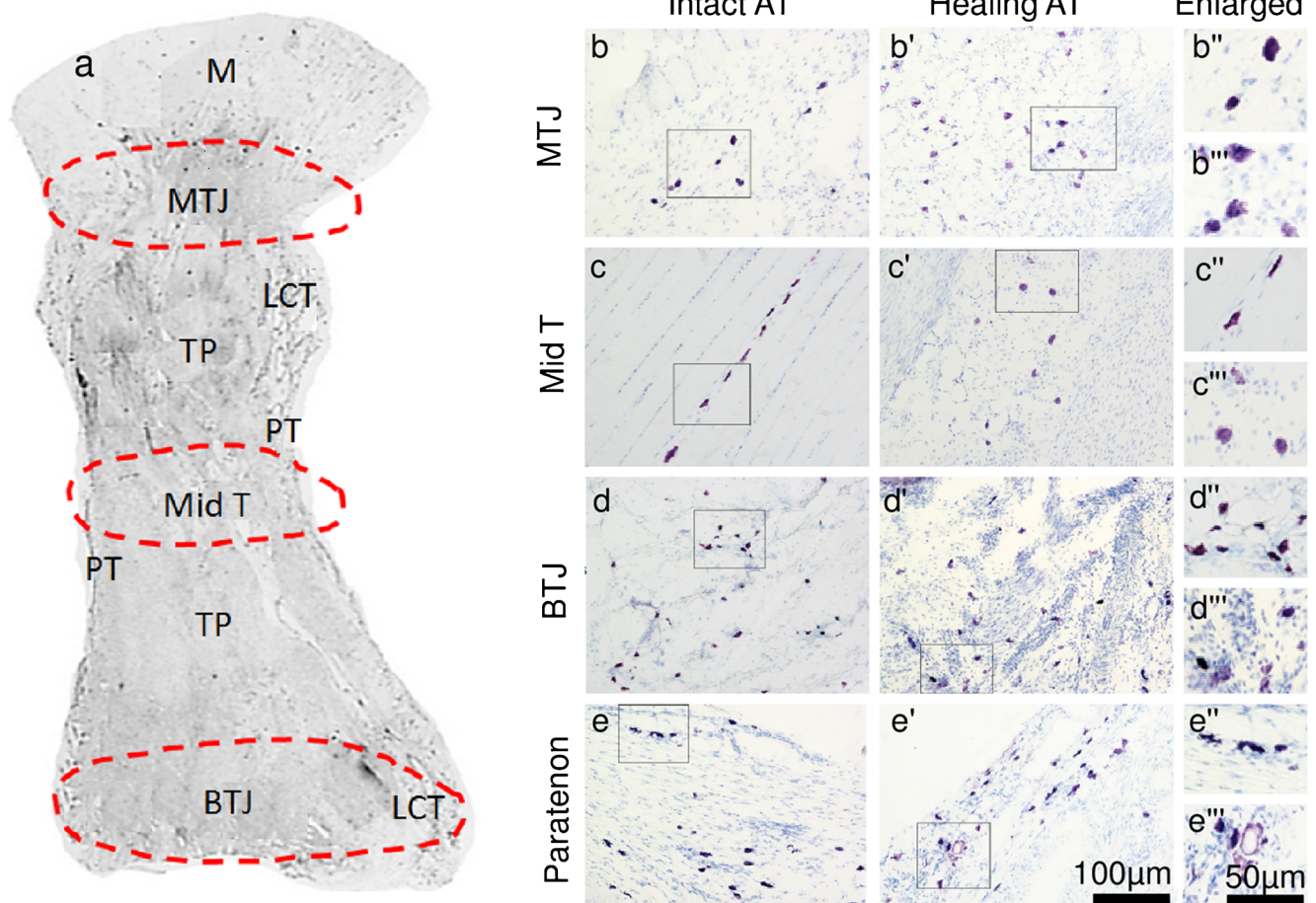


Fig. 2 Mast cell presence and degranulation in intact and healing Achilles tendon. **a** Anatomical overview of a toluidine blue-stained rat Achilles tendon, with *dotted ovals* highlighting the areas of interest. The rupture was performed in the mid-tendon region (0.5 cm from calcaneal bone). **b** Toluidine blue staining of the indicated regions of intact or healing Achilles tendon at 3 weeks post-rupture. Magnified portions of

the images are shown to the right. Note the presence of MCs in both the intact and healing tendon. Note also that MCs of healing tendon show signs of activation, as evidenced by degranulation. *Scale bars* 100 and 50 μm . Original magnifications $\times 100$ and $\times 200$. *MTJ* muscle–tendon junction, *Mid T* mid-tendon, *BTJ* bone–tendon junction, *AT* Achilles tendon, *LCT* loose connective tissue, *M* muscle, *PT* paratenon

activation (Fig. 2b; left). Notably, the MCs present in the mid-tendon showed a typical linear arrangement (Fig. 2b). In the ruptured tendon, MCs were also found in all different regions of tendon, i.e., MTJ, mid-tendon, BTJ and in the paratenon regions and were frequently found in the vicinity of blood vessels (as highlighted in the lower right panel of Fig. 2b). Whereas MCs of the intact tendon were mainly intact, MCs in the healing tendon were frequently degranulated. This was observed in all of the assessed regions.

Next, the total numbers (intact + degranulated) of MCs in the different regions of the control and ruptured tendon, as well as their state of activation (degranulation) were quantified. As shown in Fig. 3a, the total numbers of MCs were significantly higher in ruptured versus intact MTJ, mid-tendon and BTJ regions.

To assess the activation state of the MCs, the number of non-degranulated (ND), partially degranulated (PD) and extensively degranulated (ED) MCs were counted (Fig. 3b). These analyses revealed that MCs of both the MTJ, mid-tendon and BTJ were predominantly in a non-activated (non-degranulated) state in the intact tendon (Fig. 3c, left panel). In contrast, MCs in all these regions of the healing tendon were predominantly degranulated, i.e., showed signs of activation (Fig. 3c, right panel). In total, approximately

80% of the MCs in MTJ, mid-tendon and BTJ of the healing tendon displayed degranulation (most of which was extensive), whereas only 20% of the MCs in the intact tendon were degranulated (Fig. 3d). Collectively, these data indicate that tendon rupture is associated with extensive activation of MCs, in turn suggesting that MCs actively contribute to the processes occurring in response to Achilles tendon rupture.

Mast cell tryptase and NMDAR1 in intact and healing AT

Previous studies have shown that MCs are often localized in close proximity to nerve endings and it has also been proposed that MC–nerve interaction could be of biological relevance in pathological settings (Gottwald et al. 1998; Li et al. 2012). Based on these notions, we hypothesized that MCs of the healing tendon could potentially communicate with nerve cells, with the possibility that such communication could be of biological significance during the process of tendon healing. To address this possibility, we investigated whether MCs and nerve cells are closely localized within the healing tendon, by immunofluorescence staining for tryptase (MC marker) and NMDAR1 (nerve marker) in adjacent tissue sections. Strong staining for tryptase, using a polyclonal rabbit anti-tryptase antibody, was seen in all assessed areas of the ruptured tendon (MTJ, mid-tendon,

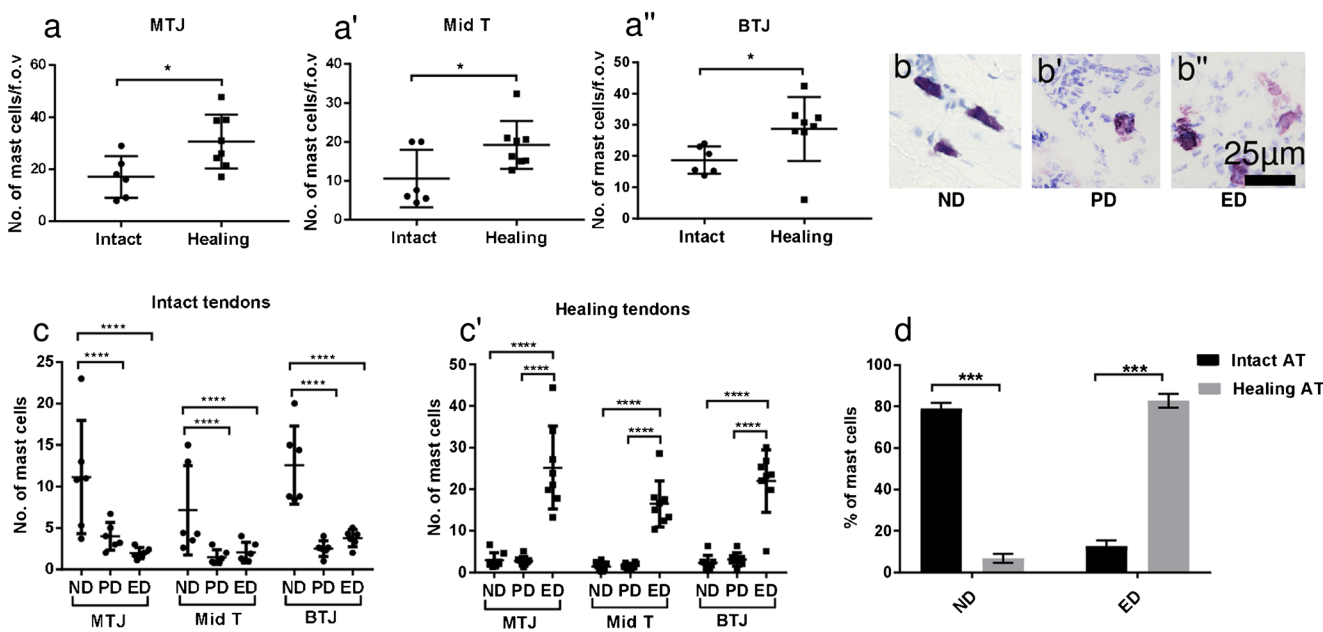


Fig. 3 Number of mast cells and the state of degranulation in intact and healing Achilles tendon. **a** Total number of mast cells in the muscle–tendon junction, mid-tendon and bone–tendon junction of intact and healing Achilles tendon, based on toluidine blue staining. **b** Representative images depicting non-degranulated (left panel), partially degranulated (middle panel) and extensively degranulated mast cells (right panel). **c** Quantification of extensively degranulated, partially degranulated and non-degranulated mast cells in the muscle–tendon junction, mid-tendon and bone–tendon junction of the intact and healing

Achilles tendon. **d** Percentage of non-degranulated, partially degranulated and extensively degranulated mast cells in the muscle–tendon junction, mid-tendon and bone–tendon junction of intact versus healing tendon. Results are given as mean ± SD (intact, *n* = 6 and healing, *n* = 8). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Scale bar 25 μm. *MTJ* muscle–tendon junction, *Mid T* mid-tendon, *BTJ* bone–tendon junction, *AT* Achilles tendon, *ND* non-degranulated, *PD* partially degranulated, *ED* extensively degranulated, *f.o.v.* field of view

BTJ), whereas somewhat weaker staining was seen in the corresponding regions of the intact tendon (Fig. 4a). Immunoreactivity for NMDAR1 was detected in all of the assessed regions of the ruptured tendon but was seen only to a limited extent in the same regions of the intact tendons. Notably, NMDAR1 staining was strong in the mid-tendon of the ruptured tissue, whereas the mid-tendon of intact tissue was essentially negative for NMDAR1, indicating that tendon healing after injury is accompanied by upregulation of NMDAR1. To ensure that the staining for tryptase and NMDAR1 was specific, the primary antibodies were replaced with negative control antibodies. Indeed, the staining was completely abrogated when using control primary antibodies, indicating that the staining for tryptase and NMDAR1 was specific (Fig. 4b). Together, these findings indicate that MCs can be found in close proximity to nerve cells in the healing tendon, in support of the hypothesis that MCs and nerve cells may communicate during tendon healing.

Co-localization of MC tryptase and NMDAR1 at the rupture site

To provide a deeper insight into the possibility that MCs and nerve cells can communicate in the healing tendon, we also performed double immunofluorescence staining for tryptase and NMDAR1. In agreement with the data derived via staining of adjacent sections (see Fig. 4), double immunofluorescence staining revealed that MCs (as marked by tryptase staining) and NMDAR1-positive cells were found closely located in the various regions of the tendon (Fig. 5). In further agreement with the staining of adjacent sections, NMDAR1 positivity was minimal in the mid-tendon of intact tendon whereas strong positivity was seen in the corresponding region of the ruptured tendon. We also noted the presence of cells that were double-positive for tryptase and NMDAR1. Such double positivity was seen in the MTJ, mid-tendon and BTJ of ruptured tendon but was only very scarcely observed in corresponding regions of the intact tendon (Fig. 5). A more detailed analysis

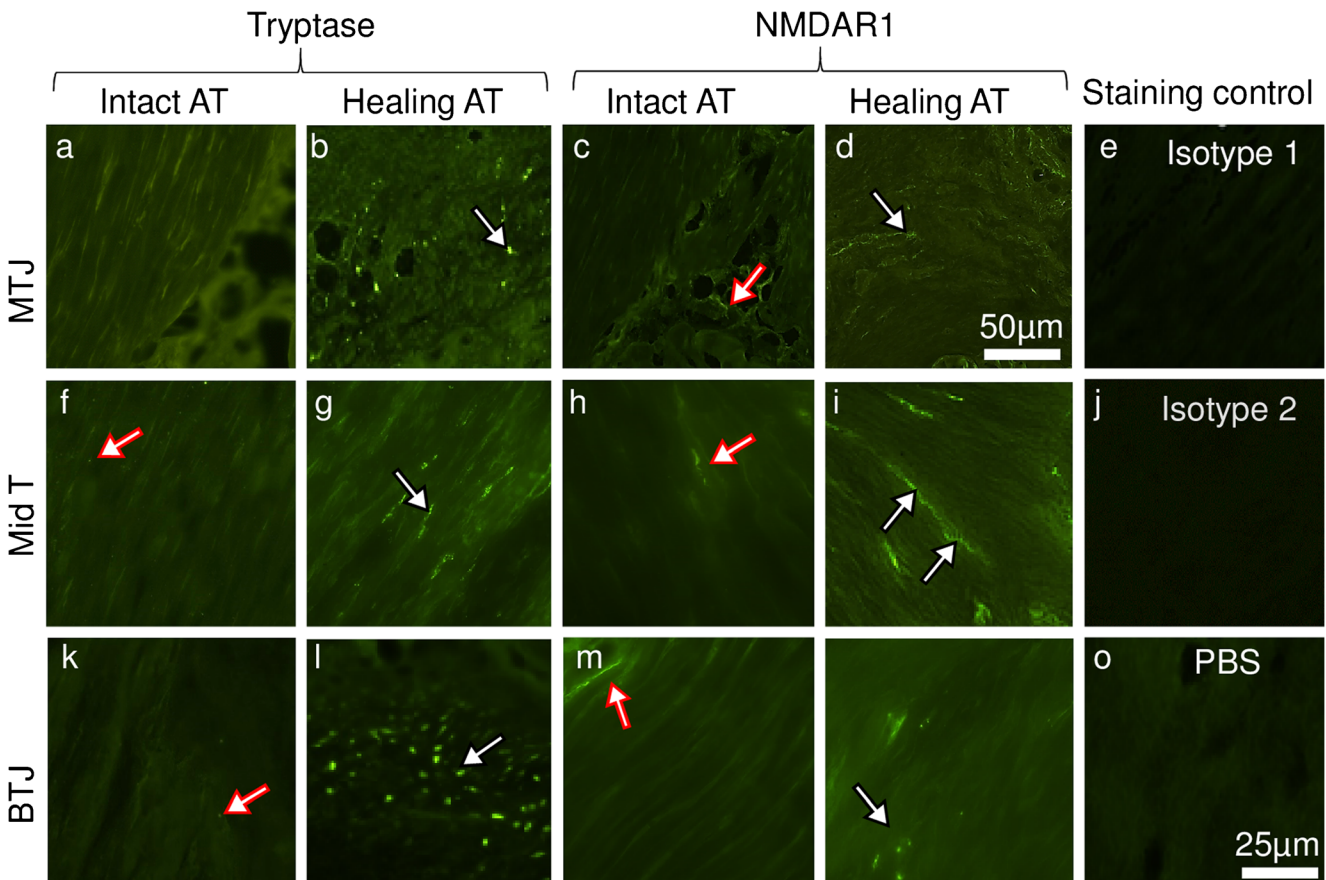


Fig. 4 Immunostaining for mast cell tryptase and NMDAR1 in intact and healing Achilles tendon. **a** Immunofluorescence micrographs of longitudinal sections from muscle–tendon junction, mid-tendon and bone–tendon junction of intact and healing Achilles tendon. Adjacent sections were stained for tryptase (*left panels*) and NMDAR1 (*right panels*). Note the increased tryptase staining in the healing tendon. Note also the increased NMDAR1 positivity of the healing versus intact mid-

tendon region. **b** Negative controls for the tryptase (rabbit IgG) and NMDAR1 (mouse IgG1), showing that the tryptase and NMDAR1 staining is specific. *Scale bars* 25 and 50 μm . *MTJ* muscle–tendon junction, *Mid T* mid-tendon, *BTJ* bone–tendon junction, *AT* Achilles tendon, *NMDAR1* N-methyl-D-aspartate (NMDA) receptor-1, *PBS* phosphate buffer saline

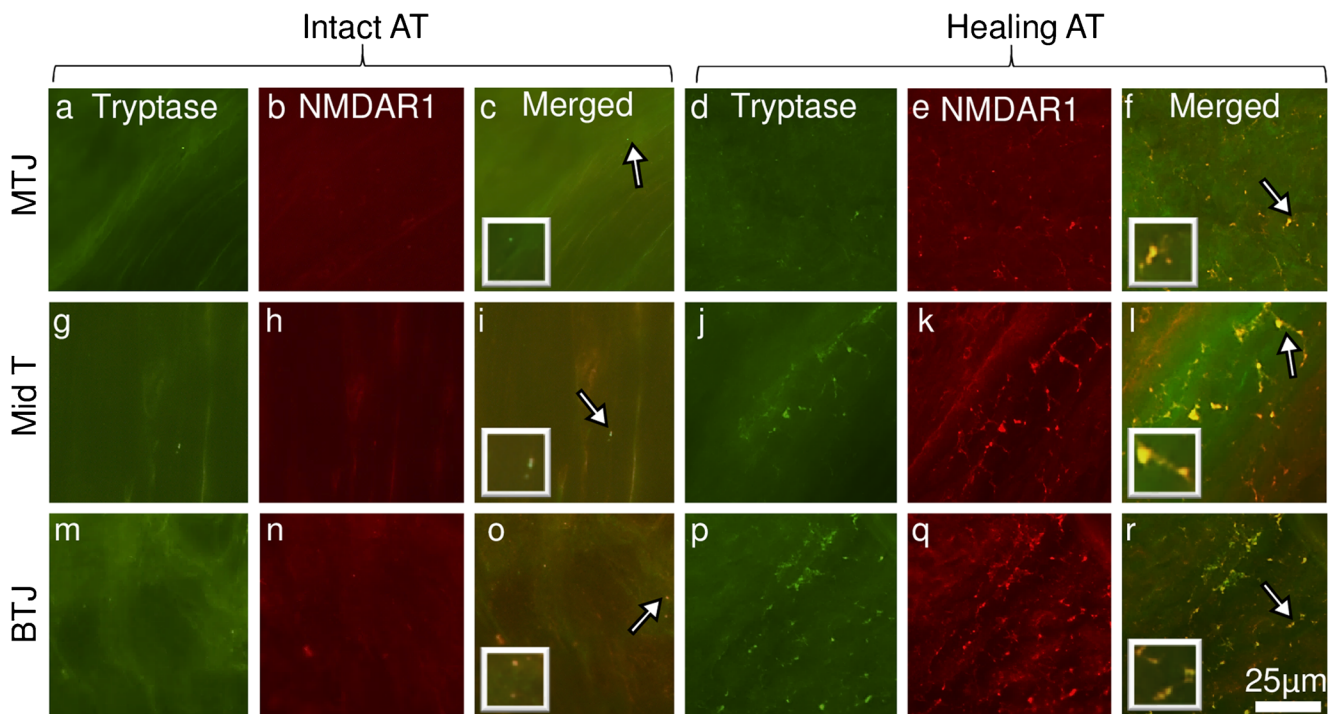


Fig. 5 Co-localization of MC tryptase and NMDAR1. Double immunofluorescence staining for tryptase (*green*) and NMDAR1 (*red*) in longitudinal sections from the muscle–tendon junction, mid-tendon and bone–tendon junction of intact and healing Achilles tendon. Note the stronger staining for both tryptase and NMDAR1 in healing vs. intact

Achilles tendon. Note also the co-localization of tryptase and NMDAR1 (magnified in the *arrowed* area) Scale bars = 25 μ m. *MTJ* muscle–tendon junction, *Mid T* mid-tendon, *BTJ* bone–tendon junction, *AT* Achilles tendon, *NMDAR1* N-methyl-D-aspartate (NMDA) receptor-1

of the callus tissue in the actual area of rupture in the tendon proper was then performed (the ruptured area was identified by tissue morphology). MCs were seen abundantly in the callus area, whereas only limited numbers were seen in the corresponding region of the intact tendon (Fig. 6). Moreover, clear co-localization of tryptase and NMDAR1 was seen in the ruptured area. Together, these findings indicate that cells of the healing tendon may co-express tryptase and NMDAR1.

Discussion

MCs have previously been implicated as detrimental players in numerous pathological settings, ranging from allergic disorders including asthma to various other types of pathologies such as cancer, autoimmune disorders and atherosclerosis (Anand et al. 2012; Wernersson and Pejler 2014). In addition, MCs have been suggested to have a number of beneficial functions, e.g., in the context of bacterial and parasite infection, as well as in wound healing (Chiu et al. 2012; Reber et al. 2015). Here, we explored a novel aspect of MCs: their possible involvement in tendon healing in the context of AT rupture.

Our data established that MCs are found in intact AT, contralateral to the ATR in this rat model. However, the numbers of MCs were increased in the healing tendon, after

experimentally-induced rupture. This increase of MCs was particularly prominent in the MTJ but was also seen in the mid-tendon and BTJ. Previous clinical studies based on samples derived from patients suffering from AT rupture have provided limited support for an increase in MC numbers during healing (Scott et al. 2008). Moreover, an increase in MC density has been suggested after rabbit deep flexor tendon repair (Berglund et al. 2010) and in the tendinopathy seen in the calcaneal tendon overuse rat model (Pingel et al. 2013). However, the present study is to our knowledge the first to document increased MC numbers in experimentally-induced tendon rupture in a rat model for Achilles tendon rupture.

A major finding of this investigation was that MCs of the healing tendon showed signs of activation, as evidenced by extensive degranulation. Notably, MC activation was prominent in all investigated areas of the healing tendon, i.e., MTJ, mid-tendon and BTJ, suggesting that tendon healing is associated with widespread activation of MCs. The consequence of MC activation in the context of tendon healing and possibly tendinopathy development, is intriguing. When MCs are activated to degranulate, they release a wide panel of preformed mediators from stores in their secretory granules (Wernersson and Pejler 2014). These mediators encompass a number of MC-restricted proteases including tryptase, chymase and CPA3 (Pejler et al. 2010). In addition, MC

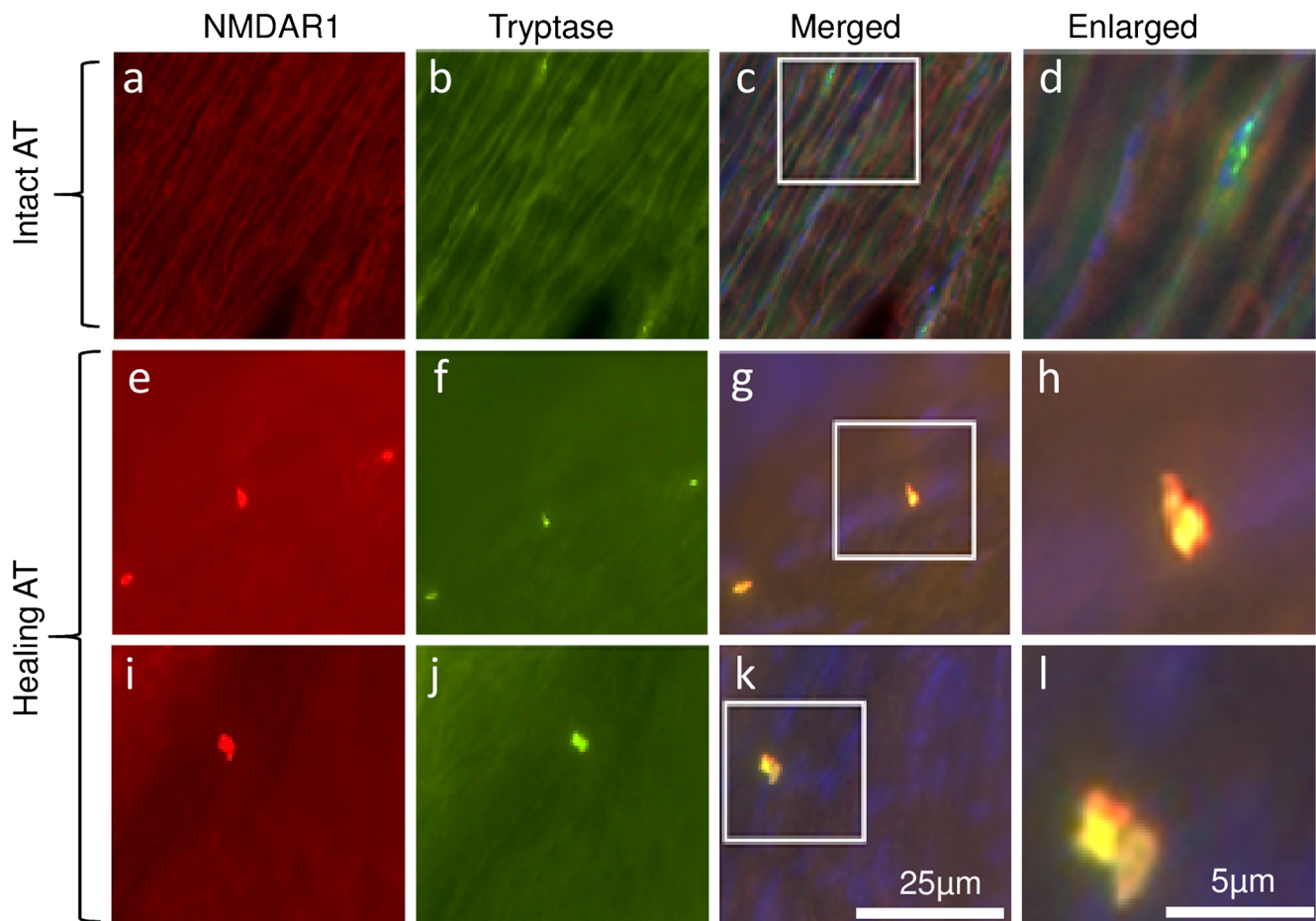


Fig. 6 Co-localization of MC tryptase and NMDAR1 at the site of rupture. Sections were taken from the site of rupture in the mid-Achilles tendon, with the corresponding region of intact Achilles tendon as control. Sections were subjected to double immunofluorescence staining for

tryptase (green) and NMDAR1 (red). Note the co-localization of tryptase and NMDAR1 at the site of rupture. Enlarged images (5 times) are shown to the right. Scale bars 5 and 25 μm . AT Achilles tendon, NMDAR1 N-methyl-D-aspartate (NMDA) receptor-1

degranulation causes the release of bioactive amines (e.g., histamine, serotonin, dopamine), proteoglycans, cytokines, growth factors (e.g., TGF- β , NGF, FGF2, VEGF) and lysosomal hydrolases (Wernersson and Pejler 2014). Potentially, several of the compounds released from activated MCs could influence the inflammatory and proliferative healing phases after tendon rupture. For example, MC-derived growth factors such as TGF- β (Nakae et al. 2007) and FGF2 could contribute to tissue remodeling processes (Maltby et al. 2009), including stimulation of collagen synthesis (Coussens et al. 1999). Further, MC-derived VEGF (Grutzkau et al. 1998) and NGF may contribute to neo-angiogenesis and nerve ingrowth (Leon et al. 1994) and MC proteases such as tryptase could activate protease-activated receptors on the surface of nerve cells (Akers et al. 2000; Saito and Bunnett 2005).

Another striking finding in this study was that NMDAR1 and tryptase appear to co-localize in the healing tendon. Tryptase is a MC-restricted protease that is well established as a specific marker for MCs (Castells and Schwartz 1988). Hence, the tryptase-positive cells most likely correspond to

MCs and, consequently, the double tryptase:NMDAR1-positive cells may represent MCs that co-express glutamate receptors. To the best of our knowledge, it has not been shown previously that MCs can express such receptors, although there is limited evidence that MCs may respond to glutamate receptor antagonists (Hamamoto et al. 2013). However, it is known from previous studies that various other types of immune cells, e.g., neutrophils, can express various glutamate receptors (Gupta et al. 2013) and the present study thus introduces the MC as a novel cell type potentially having the ability to respond to glutamate. The potential consequences of induced NMDAR1 expression in the MCs of the ruptured tendon are not clear at present. However, we may speculate that MCs expressing NMDAR1 can respond to glutamate released from afferent nerve endings, possibly inducing MC activation and in this way providing an additional amplifying loop to maintain MC activation and continuous nerve cell engagement. However, further experimental studies are needed to better evaluate the role of a MC–nerve loop.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest in relation to this work.

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