

**Macrophage polarization contributes to local inflammation and structural change in the multifidus muscle  
after intervertebral disc injury**

## Abstract

**Purpose:** Intervertebral disc (IVD) lesion and its subsequent degeneration have a profound effect on the multifidus muscle. The subacute/early chronic phase of multifidus remodeling after IVD lesion has been proposed to be regulated by inflammatory processes. The balance between pro-inflammatory (M1) and anti-inflammatory (M2) macrophages plays an important role in maintaining tissue integrity after injury. The localization, polarization of macrophage subtypes, and their mediation of the pro-inflammatory cytokine Tumor necrosis factor (TNF) are unknown in paraspinal muscles during IVD degeneration. A sheep model of IVD degeneration was used to investigate the role of macrophages and TNF in the structural alterations that occur within the multifidus muscle.

**Methods:** Anterolateral lesions were induced at L3-4 IVD in sheep. Multifidus muscle tissue at L4 was harvested three and six months after lesion and used for immunofluorescence assays to examine total macrophage number, macrophage polarization between M1 and M2, and to assess the localization of TNF expression in muscle, adipose and connective tissue from injured and naïve control animals.

**Results:** A greater proportion of M1 macrophages is present in muscle at both 3- and 6-months after IVD lesion, and adipose tissue at 6 months. Total number of macrophages is unchanged. At 6-months, expression of TNF is increased in adipose and connective tissue and the proportion of TNF expressed by M1 macrophages is increased.

**Conclusion.** These data support the proposal that macrophages and TNF (pro-inflammatory cytokine) play an active role in the subacute-early chronic phase of remodeling in muscle, adipose and connective tissue of the multifidus during IVD degeneration. This presents a novel target for treatment.

**Keywords.** Macrophage, multifidus muscle, IVD lesion, pro-inflammatory, TNF, adipose

## 1    **Introduction**

2            Low back pain (LBP) is the leading cause of disability worldwide[1,2] with enormous individual and  
3    society burden[3]. Many mechanisms explain development of LBP and its acute-to-chronic transition. Recent  
4    attention has focused on possible involvement of local (at/near tissue injury), systemic, and/or neural inflammatory  
5    processes, which can regulate structural tissue changes, peripheral nociceptor sensitization, and pain sensitivity  
6    throughout the nervous system[4-6]. LBP involves many inflammatory mediators. Some are biomarkers of LBP  
7    pathologies[5], others track disease severity[4]. Recent work highlights the potential role for inflammatory  
8    mediators in structural remodelling of paraspinal muscles following spine injury[7-9].

9            Intervertebral disc (IVD) degeneration presents in ~40% of LBP and inflammation is linked to its  
10   progression[10,11]. IVD degeneration impacts the structure and function of surrounding tissues, including muscle.  
11   Acutely after IVD injury the deep back muscle, multifidus, undergoes rapid atrophy with neural inhibition[12,13]. In  
12   the subacute/early chronic period muscle fibrosis, fatty infiltration and transformation of muscle fibers from slow-to-  
13   fast develops[8,7,14,9]. This progresses to muscle tissue atrophy with chronic degeneration[15]. These observations  
14   concur with early multifidus muscle atrophy[16] and later fat infiltration[17-20] in human studies. Animal models of  
15   IVD injury show greater gene expression of pro-inflammatory cytokines (e.g. tumour necrosis factor [TNF] and  
16   interleukin-1beta [IL-1 $\beta$ ]), in multifidus[8,7,9]. Based on other diseases, this muscle pro-inflammatory response  
17   might provide a novel mechanism regulating structural muscle remodelling[7,8] and ongoing nociceptive input[21].  
18   Major gaps remain. It is unknown what cell/tissue types are responsible for the pro-inflammatory cytokine gene  
19   expression, and whether increased gene expression translates to greater production of cytokines in multifidus.

20            After muscle injury, macrophages regulate inflammation, tissue integrity and pain[22,6,23]. M1 (pro-  
21   inflammatory) and M2 (anti-inflammatory) macrophage subtypes contribute at different times during healing.  
22   Through cytokine expression, macrophage subtypes affect collagen synthesis and other processes that regulate  
23   muscle structure[24-26]. Elimination of macrophages from muscle prevents chronic hyperalgesia[22]. Treatment of  
24   muscle pain with exercise/acupuncture alters the ratio of macrophage subtypes, increasing M2 and decreasing M1,  
25   which can prevent chronic pain[6] and reduce pain behaviours[27]. Such transformation would decrease M1-  
26   mediated expression of pro-inflammatory cytokines and increase M2-mediated expression of anti-inflammatory  
27   cytokines. Conversely, transformation from M2-to-M1 may negatively impact muscle health. In experimental IVD

injury there is no muscle injury, yet pro-inflammatory cytokine gene expression and structural remodelling occur[7-9]. Mechanistically, modified macrophage subtype and cytokine expression may provide a novel explanation for the dramatic muscle changes we have observed.

This study aimed to examine the time-course of changes in total macrophage numbers and ratios of M1/M2 subtypes in muscle and adipose tissue of multifidus muscle of sheep after IVD lesion. A second aim was to examine the distribution of TNF expression in muscle, adipose and connective tissues and whether any inter-relationship with M1 macrophages existed.

## **Materials and methods**

### **Animals**

Twenty-seven merino wether (castrated male) sheep (3-4 years) were drawn from the no-treatment control group of a study examining mesenchymal stem cell treatment of IVD degeneration[28,29]. Tissue from these animals has been used in previous studies[8,7,9]. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

### **Surgical procedure, IVD lesion, postoperative care and tissue harvesting**

Seventeen sheep (“injured” group) received a controlled 6 x 20 mm anterolateral annular lesion in the L1-2, L3-4 and L5-6 IVDs as described previously[7]. This initiates molecular and pathological degenerative IVD changes consistent with changes in humans[30]. Animals were housed in pens and closely monitored for 10 days post-surgery to ensure wound healing and absence of behaviour abnormalities. Ten age- and weight-matched sheep did not receive surgery (“control” group). Animals were cohoused in an open paddock and allowed to roam freely. The multifidus muscles adjacent to the L2, L4 and L6 spinous processes were harvested from four control and six injured animals at three months post-injury, and six control and eleven injured animals at six months. Within the injured group, tissues harvested from the side ipsilateral to the disc lesion were termed the “injured” group, whereas tissues

harvested from the contralateral side were termed the “non-injured” group. Different tissue processing protocols were used for multifidus muscle specimens from each level and suited to downstream experiments. Only muscle tissue adjacent to L4 was processed in a manner that enabled methods to be employed for this study. This tissue was fixed in 10% Neutral buffered formalin, dehydrated and embedded in paraffin then 7µm microtome transverse sections were attached to positively charged microscope slides.

#### **Fluorescent immunolocalisation of macrophages and TNF expression**

Multifidus tissue sections were rehydrated for 15min, treated with 5% hydrochloric acid for 5min and then blocked in 5% Bovine Serum Albumin in Tris-Buffered saline (TBS) for 2h. Tissue sections were incubated overnight (4°C), with anti-CD11c (1:100, Abcam, AB33483), anti-CD206 (1:400, Abcam, AB64693) and Anti-S100A9 + Calprotectin (S100A8/A9 complex) (1:400, Abcam, AB22506) primary antibodies to identify macrophages. Following rinsing in TBS goat anti-Armenian Hamster 647 (1:200, Abcam), donkey anti-rabbit 594 (1:200) and goat anti-mouse 488 (1:200, Abcam) secondary antibodies were added for 1h (room temperature). For TNF immunolocalisations, tissue sections were treated with anti-TNF (1:2000, Abcam) for 4 days (4°C) and double labelled with anti-CD11c (1:1000, Abcam) to identify M1 macrophages. Sections were treated with goat anti-Armenian Hamster 647 (1:400, Abcam) and donkey anti-rabbit 594 (1:400) secondary antibody for 3h (room temperature) for visualisation. All sections were mounted with DAPI mounting media (Abcam) to stain cell nuclei. All experiments were performed with a no primary antibody control.

#### **Morphometric image analysis of the spatiotemporal localisation of macrophage populations**

A fluorescent microscope with digital camera (Axio Microscope, Zeiss) was used to analyse the macrophage subtypes in the medial half of the deepest third of the multifidus muscle, which is the region shown to display modified cytokine gene expression[7-9]. First, areas of adipose and muscle tissue were identified from the whole muscle image under conventional light microscopy. Second, three separate areas were selected for analysis for each tissue under higher magnification (20x magnification 430 x 320µm) [7] (see Fig. 1 for identification of tissue type and example sample areas). This analysis was not conducted for connective tissue as autofluorescence present in a subpopulation of cells located in the connective tissue, precluded analysis of macrophage subtype in this component of the analysis. Third, macrophage subtypes (M1/M2) were identified when cells were co-stained by the total macrophage marker and either anti-CD11c (M1-marker) or anti-CD206 (M2-marker) primary antibodies.

M1/M2 and total macrophage numbers were counted in each image and their totals combined to reveal the total number and proportion of M1/M2 macrophages (ImageJ, NIH).

#### ***Quantitation of TNF expression by immunolocalised macrophage populations using morphometric image analysis***

TNF and anti-CD11c (M1-marker) were co-localised (see above). For this analysis, three areas each of muscle, adipose and connective tissues were identified in the deepest third the multifidus as described above at 20x magnification (Fig. 1). For morphometric image analysis calculations, TNF was quantified (ImageJ, NIH) by measuring the integrated density of each image and subtracting the mean grey value of the background multiplied by the image area[31]. The proportion of TNF that co-localized with M1 macrophages was determined by separating the TNF signal that co-localized with CD11c from the total TNF signal using the “image calculator” function of ImageJ. Data are presented as a proportion of total TNF expression.

#### **Statistical Analysis**

Macrophage number, proportion and TNF signal were compared between the muscles of control animals and the injured and non-injured sides of injured animals, using one-way ANOVAs at three- and six-months. Post-hoc analysis involved Duncan’s multiple range test. Pearson’s correlation examined the relationship between TNF expression and the M1 macrophage proportion. Results were interpreted as: weak- $r < 0.35$ ; moderate- $r = 0.36–0.67$ ; strong- $r = 0.68–0.90$ ; or very strong- $r > 0.90$ . Significance was set at  $P = 0.05$ .

#### **Methodological considerations**

The animals used in this study were also involved in another study to investigate the role of mesenchymal stem cell injection in IVD degeneration[28,29]. Only the animals without stem cell treatment were used in this study. The lesion group animals in our study received an injection of phosphate buffered saline (used as a sham control for the aligned stem cell study) into the IVD on the contralateral side to the lesion side, at either 4- or 12-weeks post-surgery. No statistical differences were detected between the 4- and 12-week injection groups and were therefore pooled for analysis. Control animals in the present study did not receive an injection. The injection is not thought to contribute to IVD degeneration and the subsequent alterations to the multifidus because of the absence of early structural changes to the multifidus muscle on the injected side Because of issues antibody availability for

ovine muscle, several analyses were not possible. These include analysis of localization of IL-1b expression and immunolocalisation of M2 macrophages with cytokine expression. These questions should be answered in future studies.”

## **Results**

Table 1 shows the summary of all statistical analyses of macrophage numbers and M1/M2 ratios and TNF expression.

### **Total macrophage and macrophage subtypes**

**Three months:** Although total macrophage number in muscle (Fig. 2) and adipose (Fig. 3) did not differ between control and injured animals at three months, there was a significantly greater proportion of M1, and lower proportion of M2 macrophages in muscle from the injured and non-injured sides than control (Table 1;Figs 1/3). Proportion of M1/M2 was unchanged in adipose (Figs 2/4).

**Six months:** At six months after IVD lesion the total number of macrophages was unchanged in muscle (Fig. 2) and adipose (Fig. 3). The proportion of M1 macrophages in muscle was greater on the injured side than the controls and non-injured side (Table 1;Figs 1/3). Unlike three months, proportions of M1/M2 macrophages were greater and lower in adipose, respectively, in the injured side than the non-injured side and controls (Table 1;Figs 2/4).

### **TNF expression**

**Three months:** Immunohistochemistry did not reveal a detectable change of TNF expression following IVD lesion in muscle, adipose or connective tissue at three months (Table 1;Fig. 6).

**Six months:** Although TNF expression in muscle tissue was not affected by IVD lesion at six months (Fig. 6), TNF expression in adipose was increased on the injured, but not non-injured side (Fig. 6). IVD lesion increased the TNF expression in connective tissue from the injured side (Fig. 6).

### **M1 macrophage and TNF**

**Three months:** The proportion of TNF expression that co-localized with M1 macrophages in the injured animals did not differ between control and injured animals in either muscle or adipose tissue (Table 1;Fig. 7) at three months. TNF expression moderately correlated with the proportion of M1 macrophages in muscle ( $r=0.42$ ;  $P=0.14$ ) and adipose ( $r=-0.54$ ;  $P=0.07$ ; Fig. 7), but was not statistically significant.

**Six months:** In adipose, the proportion of TNF expression associated with M1 macrophages was significantly greater on the injured side than controls (Table 1;Fig. 7). There was no significant difference between the non-injured side and controls in adipose at six months. TNF expression strongly correlated with M1 macrophages in adipose ( $r=0.73$ ;  $P<0.001$ ). TNF expression associated with M1 macrophages in the muscle was similar between the control and injured groups (Fig. 7) with a weak correlation between TNF and M1 macrophage proportion ( $r=0.34$ ;  $P=0.21$ ).

## Discussion

These data provide new understanding of the nature, mechanism and timeline for inflammatory changes in the multifidus muscle after experimental injury of an adjacent IVD lesion. A transition towards the pro-inflammatory M1 macrophages in muscle at three and six months, and adipose tissue at six months, coincides with the previously reported increase in TNF gene expression[8,7,9]. Co-localised TNF protein expression with M1 macrophages and the strong correlation, suggest TNF expression in the multifidus muscle after IVD lesion is mediated by M1 macrophages in adipose tissue.

### M1 macrophages and multifidus structural remodeling

M1/M2 macrophage populations are dynamically balanced in skeletal muscle. The surrounding microenvironment regulates transitions between subtypes[32]. During IVD degeneration the multifidus muscle is constantly remodeling[8,7,9,14] and if elements of this remodeling begin before the macrophage transition this could provide a candidate mechanism to underpin this process. One possibility is the muscle fiber type transformation from slow (oxidative) to fast (glycolytic) that is significant by six months after IVD lesion[8,7], with a non-significant tendency at three months (19.8% lower slow fibers proportion than controls)[8,14]. Altered skeletal muscle microenvironment secondary to slow muscle fiber loss could affect the macrophage population and



1 hyperalgesia[23,33,34]. Slow fiber loss reduces oxidative metabolism and fatigue resistance (as observed in  
2 humans[35]), leading to increased glycolytic metabolism and lactic acid production. These alterations would reduce  
3 muscular pH, which causes hyperalgesia and pain in humans[36,37]. Removal of muscle macrophages prevents  
4 hyperalgesia induced by fatiguing stimuli [23]. The temporal relationship in our data supports the hypothesis that  
5 muscle fiber type transformation contributes to the pro-inflammatory state of macrophages following IVD lesion  
6 (Fig. 8).

7         Within an injured muscle, macrophages are important for phagocytosis of necrotic muscle fibers and  
8 regeneration of new muscle fibers[38]. M1 and M2 have specialised roles in muscle regeneration[39,40]. A transient  
9 M1 increase after muscle injury induces satellite cell proliferation and apoptosis of fibroblast progenitor cells[41].  
10 This is normally followed by a shift towards M2 macrophages, which promotes fusion of myoblast, hypertrophy of  
11 myotubes[39] and remodelling of extracellular matrix in connective tissue[42]. Abnormal ratios of the pro- and anti-  
12 inflammatory macrophages in muscle play a role in pain and structural remodelling[27,6,25,43].

13         Persistence of M1 macrophages past their transient active phase is proposed in numerous inflammatory  
14 myopathies[25,43,26]. Pro-inflammatory macrophages are enriched in the skeletal muscle of mouse models of  
15 muscular dystrophy and are actively involved in the characteristic remodelling, atrophy and fibrosis[25,43].

16         Here we provide the first evidence of elevated M1 macrophages in multifidus in our animal model of IVD  
17 degeneration, which unlike the aforementioned conditions, does not involve primary muscle pathology or direct  
18 muscle injury. The increase in M1 macrophages present in this model is between one-third to two-thirds of the  
19 observed increase in models with direct muscle pathology [27,6] .

20         Further, there are no histological or molecular signs of multifidus muscle fiber atrophy during this sub-  
21 acute/early chronic phase[7]. TNF potently regulates muscle atrophy[44] and the absence of increased TNF  
22 expression in the multifidus muscle tissue could explain the absence of muscle atrophy up to six months in animal  
23 models [8] and sub-acute period in humans [19]. Elevated M1 macrophages might regulate the muscular  
24 atrophy[15,20] observed in chronic IVD degeneration through its downstream mechanisms[8].

25         Macrophages in adipose tissue contribute to systemic inflammation and have a role in obesity, insulin  
26 sensitivity and type-2 diabetes[45]. Their role in musculoskeletal conditions is unknown. Rotator cuff tears produce

1 fatty accumulation, muscle fiber type transformation, and fibrosis[46,47]. Macrophages infiltrate into areas of fatty  
2 degeneration within the first month after injury[46]. Although macrophage subtype in the acute phase is unknown,  
3 gene expression of M1 macrophage markers is enhanced in muscle from animals with chronic rotator cuff injury[47]  
4 suggesting a prolonged inflammatory state of muscle with fat and macrophage accumulation. We extend these  
5 findings by showing IVD degeneration causes muscle fiber type transformation, fatty infiltration, and fibrosis six  
6 months after IVD lesion[8,9] in association with increased M1 macrophage proportion in muscle and adipose and  
7 increased TNF expression in adipose. These new data indicate that multifidus adipose tissue is in a pro-  
8 inflammatory state. This is of great significance when paired with the increased multifidus adipose tissue present in  
9 humans with IVD degeneration/herniation [19,20]. Increases to pro-inflammatory adipose tissue could drive the  
10 spectrum of structural changes present in the multifidus muscle in association with IVD degeneration (Fig. 8). We  
11 observed no difference in M1 proportion between injured and uninjured sides at 3 months, but a greater M1  
12 proportion on the injured side at 6 months. This may imply that paracrine effects of IVD injury increase over time,  
13 with greater impact on the side closest to the lesion. Alternatively, this may be a positive feedback loop between  
14 macrophage polarisation and TNF expression, which is only elevated on the injured side and only at 6 months.

15         TNF in adipose tissue negatively regulates the expression of peroxisome proliferator-activated receptor  
16 gamma (PPAR- $\beta/\delta$ )[48]. PPAR- $\beta/\delta$  is anti-inflammatory and suppresses macrophage infiltration[49]. PPAR- $\beta/\delta$   
17 induces a fast-to-slow fiber type transformation in skeletal muscle[50]. Its inhibition by TNF in multifidus, could  
18 contribute to slow fiber loss[8,7,9] in a vicious cycle. In the rotator cuff model, fatty accumulation increases the  
19 expression of members in the autophagy pathway[51,52]. As TNF regulates muscle autophagy[53], increased  
20 adipose and M1-mediated TNF expression in our model, suggest that the pro-inflammatory state of adipose tissue  
21 may play a role in muscular atrophy that occurs in chronic LBP[15](Fig. 8).

22         The temporal relationship between M1/M2 macrophages during muscle repair may provide clues as to their  
23 function in this process. Perturbations to M1/M2 ratio induce fibrosis throughout multiple systems[54]. The M2  
24 subtype is associated with fibrosis due to its expression of transforming growth factor beta1 (TGF- $\beta$ 1), which is  
25 expressed by macrophages in chronic inflammation. TGF- $\beta$ 1 simultaneously inhibits apoptosis of progenitor cells  
26 and promotes their differentiation into matrix producing cells[42]. In our model, TGF- $\beta$ 1 correlates with Collagen-1  
27 expression[8]. M1 macrophages and their cytokine TNF are thought to play conflicting roles in regulating fibrosis

formation[54]. Treatments that reduce muscle fibrosis, also reduce TNF expression[55-57]. Conversely, TNF acts in an anti-fibrotic role by inducing apoptosis of fibrotic precursor cells[42]. Although our data support the notion that macrophages and their cytokines (TNF/TGF- $\beta$ 1) regulate multifidus fibrosis during IVD degeneration, further examination of their roles is required.

## **Inflammation and hyperalgesia in multifidus**

Macrophages and TNF play a role in muscle hyperalgesia[21,27,22,6]. Bias towards M1 macrophages is present in mouse models of hyperalgesia[27,6]. This could be mediated by TNF, which induces hyperalgesia when injected into muscle[21]. Furthermore, using a mouse model of IVD degeneration, we have recently shown that exercise can reduce levels of TNF expression in the multifidus muscle in conjunction with reduced pain behaviors (James et al, 2017, unpublished data). It is tempting to speculate that reduced TNF expression in the multifidus in that model contributes to reduced pain behaviors as the inhibition of TNF, with Etanercept, reduces pain associated with the IVD for up to 8 weeks in humans [58]. The analgesic effects of exercise [27,6] might be mediated by regulation of the balance between M1/M2 macrophages[27,6] and it increases M2 macrophage proportion[6]. These effects are mediated by the anti-inflammatory cytokine IL-10; IL-10 receptor blockade and genetic deletion of IL-10 prevents the analgesia from these treatments[27,6]. Exercise is a common LBP treatment[59] and reduce pain associated with IVD degeneration[60]. Our data imply that these treatments effects may be partly explained by promotion of M2 macrophages over pro-inflammatory M1 macrophages.

## **Model and conclusions**

These data provide new insight into the timeline of multifidus structural remodeling after IVD injury that we proposed recently[8]. Structural changes differ between phases after injury. A sub-acute/early chronic phase of multifidus remodeling is characterized by fibrosis, fatty infiltration, muscle fiber-type transformation, and local inflammation. The new data reveal novel inflammatory mechanisms with increased M1 macrophage proportion and elevated TNF expression. The temporal relationship between macrophage/cytokine changes and structural changes during this period support their role in regulating multifidus muscle changes (Fig. 8). Our new data suggest that slow muscle fiber loss could induce the transition to M1 macrophages and TNF expression as a consequence of muscle fiber-mediated alteration to the muscle microenvironment. Whether similar mechanisms are active in humans has not been determined, however, this is plausible as IVD degeneration in humans has been shown to have some

1 similarities to the pathology observed in our model[30]. Of note, the present observations provide evidence that IVD  
2 can cause muscle changes but does not exclude the possibility the converse that muscle changes may IVD  
3 degeneration. If the present findings are confirmed in humans, this would have implications for the treatment of  
4 multifidus remodeling in LBP. Early implementation of treatments targeting the fiber-type transformation (e.g.  
5 exercise paradigms that favor slow muscle fibers[61]) might prevent local inflammation and the subsequent fatty  
6 infiltration and fibrosis. In later phases, exercise might suppress M1 and favor M2 macrophage populations[6] to  
7 break the cycle. Initial muscle fiber transformation is unlikely to be mediated by inflammation, but might result from  
8 muscle activation changes secondary to pain and/or injury (Fig. 8). The potential efficacy of treatments to address  
9 the novel targets identified in this study warrant further investigation.

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## Figure legends

**Fig. 1** A representative section of the multifidus muscle, with a Van Giesens's stain. For analysis, three randomly selected images were taken at a 20x magnification of each tissue type. Dashed lines delineate the medial and lateral halves and the deepest third of the multifidus. Images of muscle (M) and adipose (A, delineated by dotted line) were obtained from the medial half of the deepest region of the multifidus. While images of connective tissue (C, delineated by dotted line) were obtained from the lateral half of the deepest region of the multifidus.

**Fig. 2** Group data of macrophage analysis in multifidus muscle of control and IVD lesion (injured and non-injured sides) groups. Total macrophage number in muscle (top panel) and proportion of M1 and M2 macrophages in the multifidus (bottom panel) at 3 and 6 months are shown. Data are presented as mean and SD. \* $P < 0.05$ , \*\*\* $P < 0.001$  relative to control group.

**Fig. 3** Group data of macrophage analysis in adipose tissue for control and IVD lesion (injured and non-injured sides) groups. Total macrophage number in muscle and the proportion of M1 and M2 macrophages in the multifidus muscle are shown at 3 and 6 months. Data are presented as mean and SD. \* $P < 0.05$ , \*\*\* $P < 0.001$ , relative to control group.

**Fig. 4** Macrophage analysis in muscle tissue for control and injured group. Representative images of M1 (**top panel**, shown in red) and M2 (**bottom panel**, shown in red) and total (shown in green) macrophage staining in muscle. Yellow macrophages (yellow arrows; \$), reveal co-localization of either M1 (**top panel**) or M2 (**bottom panel**) markers and the total macrophage marker. White arrows (#) identify examples of macrophages that stained positive for total macrophage but not the M1 (**top panel**) or the M2 (**bottom panel**) markers. The far right panel shows the highlighted area of the merged image at higher magnification.

**Fig. 5** Macrophage analysis in adipose tissue for control and IVD lesion (injured and non-injured sides) groups. Representative images are shown for M1 (**top panel**, shown in red) and M2 (**bottom panel**, shown in red) and total (shown in green) macrophages staining in adipose. Yellow macrophages (yellow arrows; \$), reveal co-localization of either M1 (**top panel**) or M2 (**bottom panel**) markers and the total macrophage. White arrows (#) identify an example macrophage that stained positive for total macrophage but not the M1 (**top panel**) or the M2 (**bottom panel**) markers. The far right panel shows the highlighted area of the merged image at higher magnification.

**Fig. 6** TNF expression analysis in muscle, adipose and connective tissue for control and injured groups. Representative images of the association between TNF expression (green) and M1 macrophages (red) in adipose tissue (top panel). Co-localization of TNF expression and M1 macrophages is seen in yellow (yellow arrows; \$). Bottom panel shows group data of levels of TNF expression analysis in muscle, adipose and connective tissue at 3 and 6 months. All data are presented as fold difference relative to control plus SD. \*P<0.05, \*\*\*P<0.001, relative to control group.

**Fig. 7** Group data of TNF expression analysis in muscle, adipose and connective tissue from control and IVD lesioned (injured and non-injured sides) groups. Correlations between TNF expression and M1 proportion in adipose (upper panels) and muscle (middle panels) are shown at 3 and 6 months. Bottom panels show the proportion of TNF expression that is co-localized with M1 macrophages in muscle and adipose tissue. All data are presented as mean and SD. Individual  $r^2$  and P values are shown in each graph. \*P<0.05, relative to control group.

**Fig. 8** Proposed model of the timeline and mechanisms underlying the structural and inflammatory changes in the multifidus muscle after IVD lesion. The proposed three phases, acute (top), subacute-early chronic (middle) and chronic (bottom), are characterized by different structural and inflammatory changes within the multifidus muscle. These alterations (boxes) and their potential interactions (arrows) with each other are shown in their respective phases. TNF - Tumour Necrosis Factor; IL-1 $\beta$  – Interleukin-1 $\beta$ .

## Tables

**Table 1.** Results of statistical analysis of macrophage number and proportions, and TNF expression. P-values are shown.

	Three months		Six months	
	ANOVA	Post-hoc	ANOVA	Post-hoc
<b><i>Macrophage analysis in muscle tissue</i></b>				
Total number	0.922		0.57	
M1/M2 proportion	0.018	C vs I: 0.035 C vs NI: 0.042	0.0002	C vs I: 0.0002 I vs NI: 0.032
<b><i>Macrophage analysis in adipose tissue</i></b>				
Total number	0.469		0.325	
M1/M2 proportion	0.226		0.0005	C vs I: 0.0004 I vs NI: 0.027
<b><i>TNF expression</i></b>				
Muscle Tissue	0.521		0.192	
Adipose Tissue	0.564		0.0003	C vs I: 0.0002
Connective Tissue	0.219			C vs I: 0.0491
<b><i>Co-localized M1 and TNF expression</i></b>				
Muscle Tissue	0.631		0.788	
Adipose Tissue	0.378		0.044	C vs I: 0.039

ANOVA - Analysis of variance; TNF - Tumour necrosis factor; C – control animals; NI – non-injured side of injured animals; I –injured side of injured animals. M1 – M1 subtype macrophage; M2 – M2 subtype macrophage.

1   **Figures**

2   **Fig. 1**

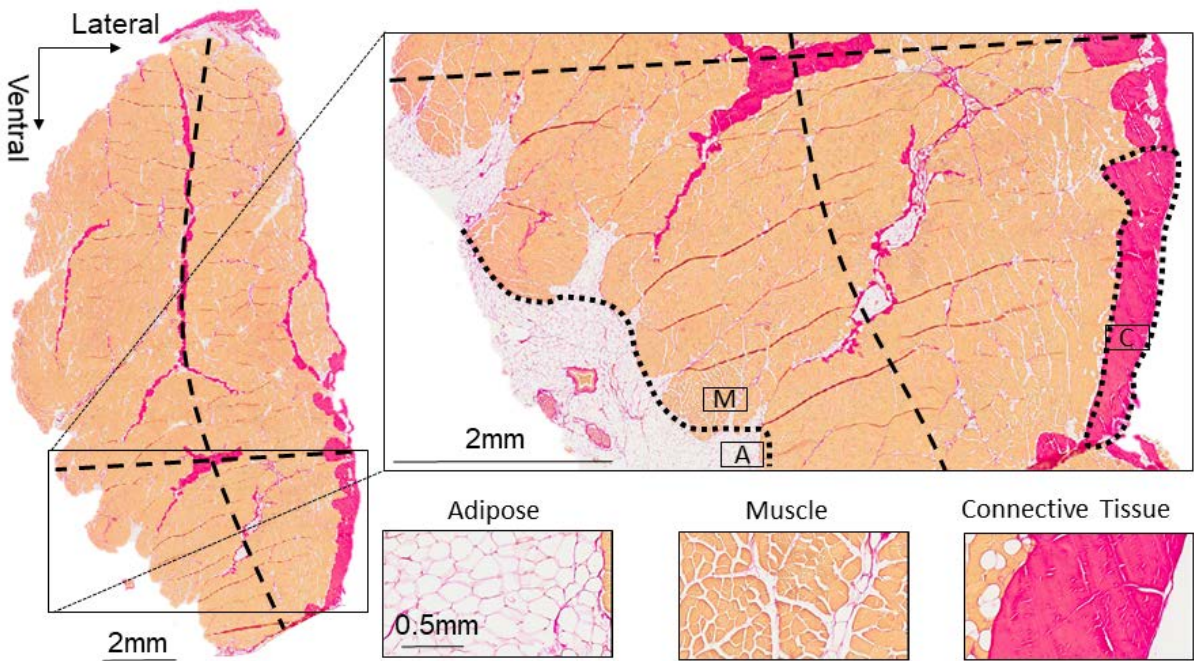


Fig. 2

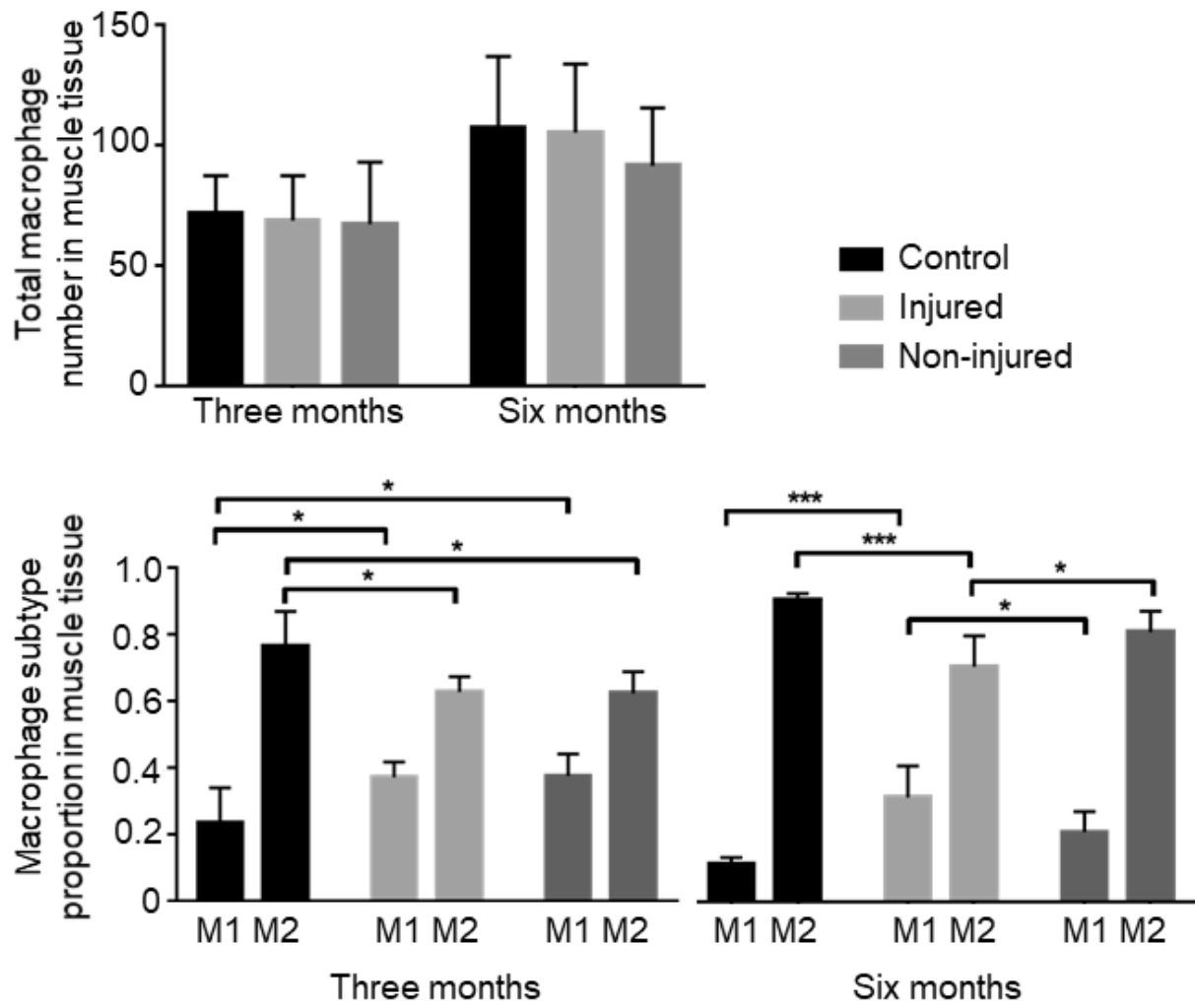


Fig. 3

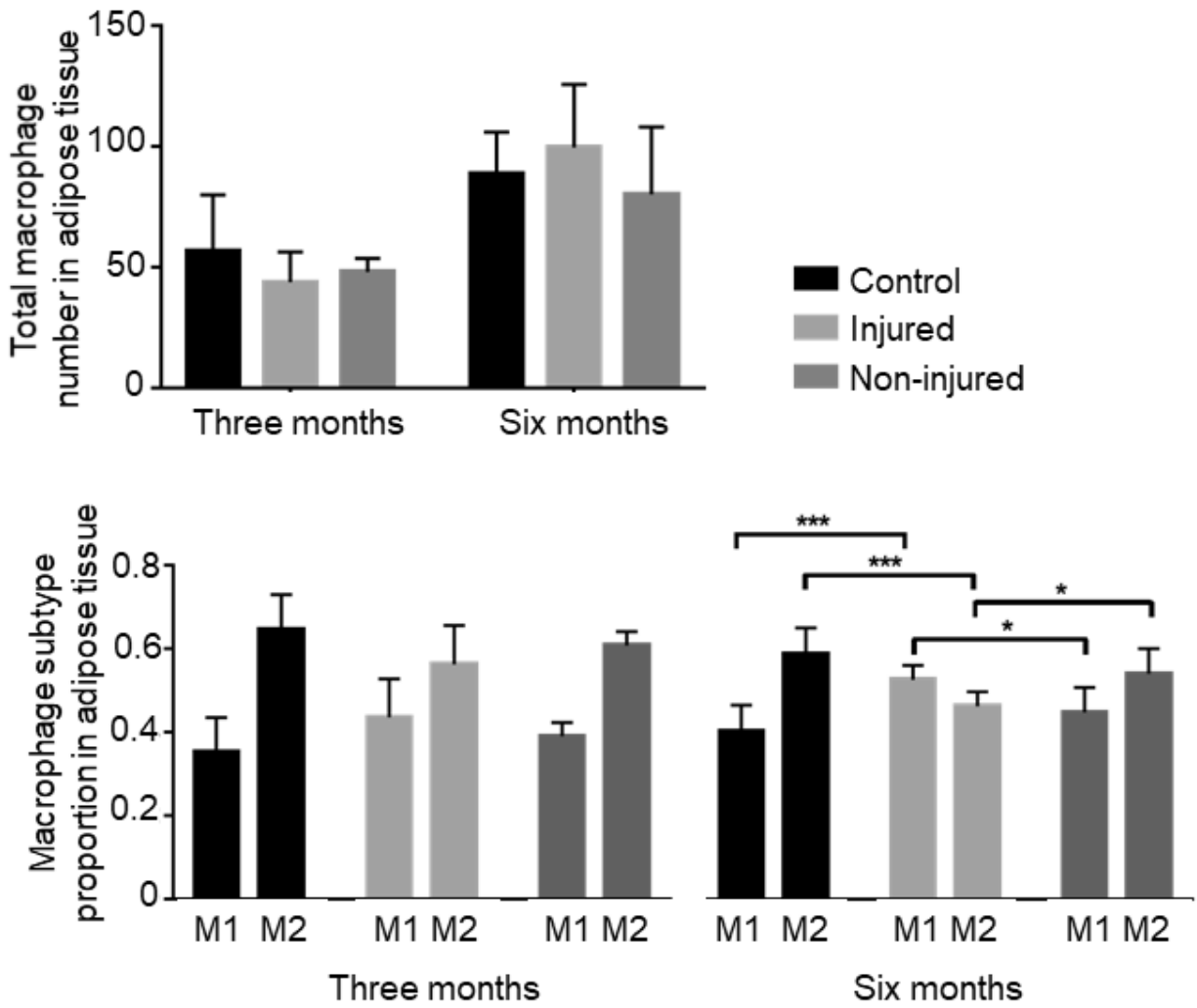
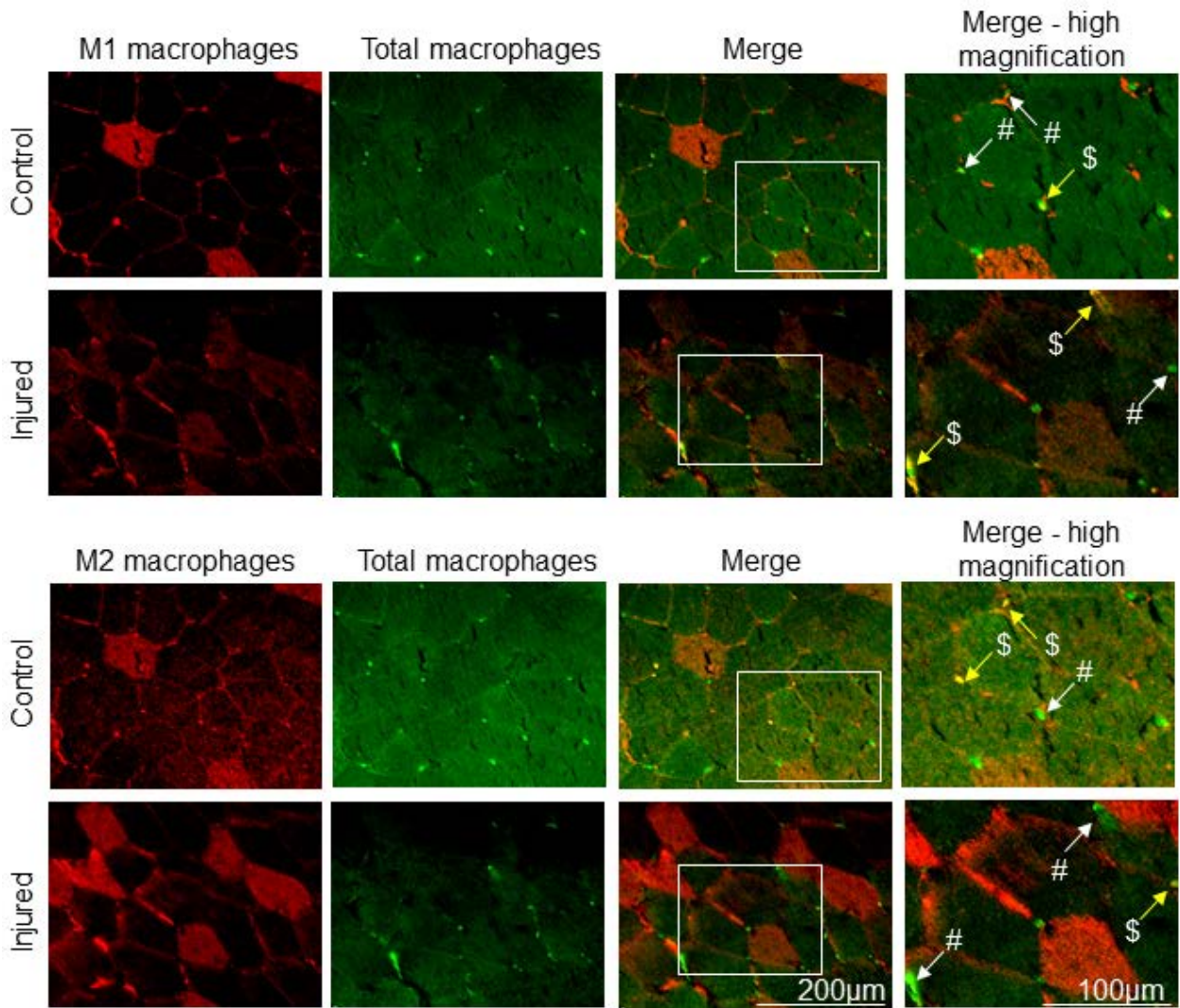
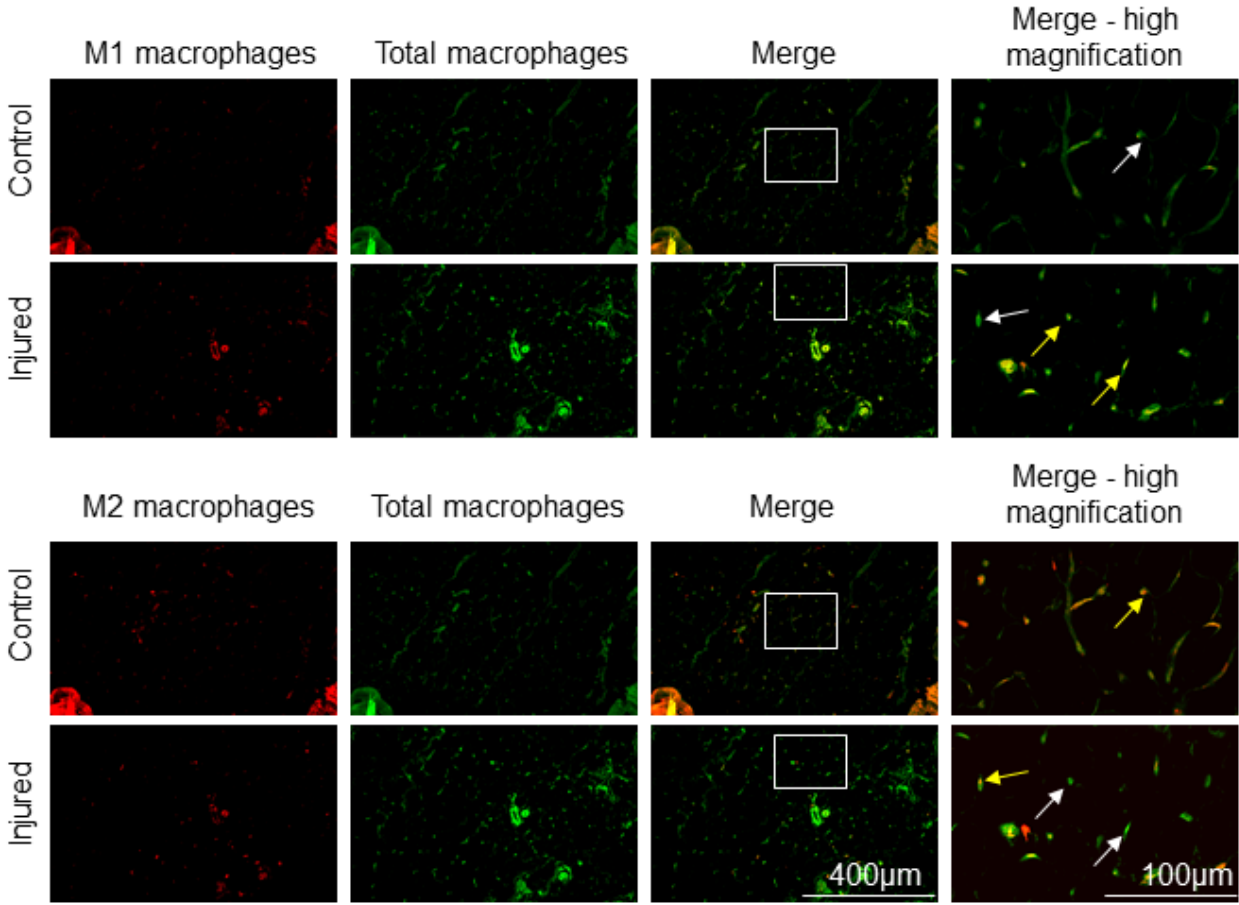


Fig. 4



1    **Fig. 5**



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1 Fig. 6

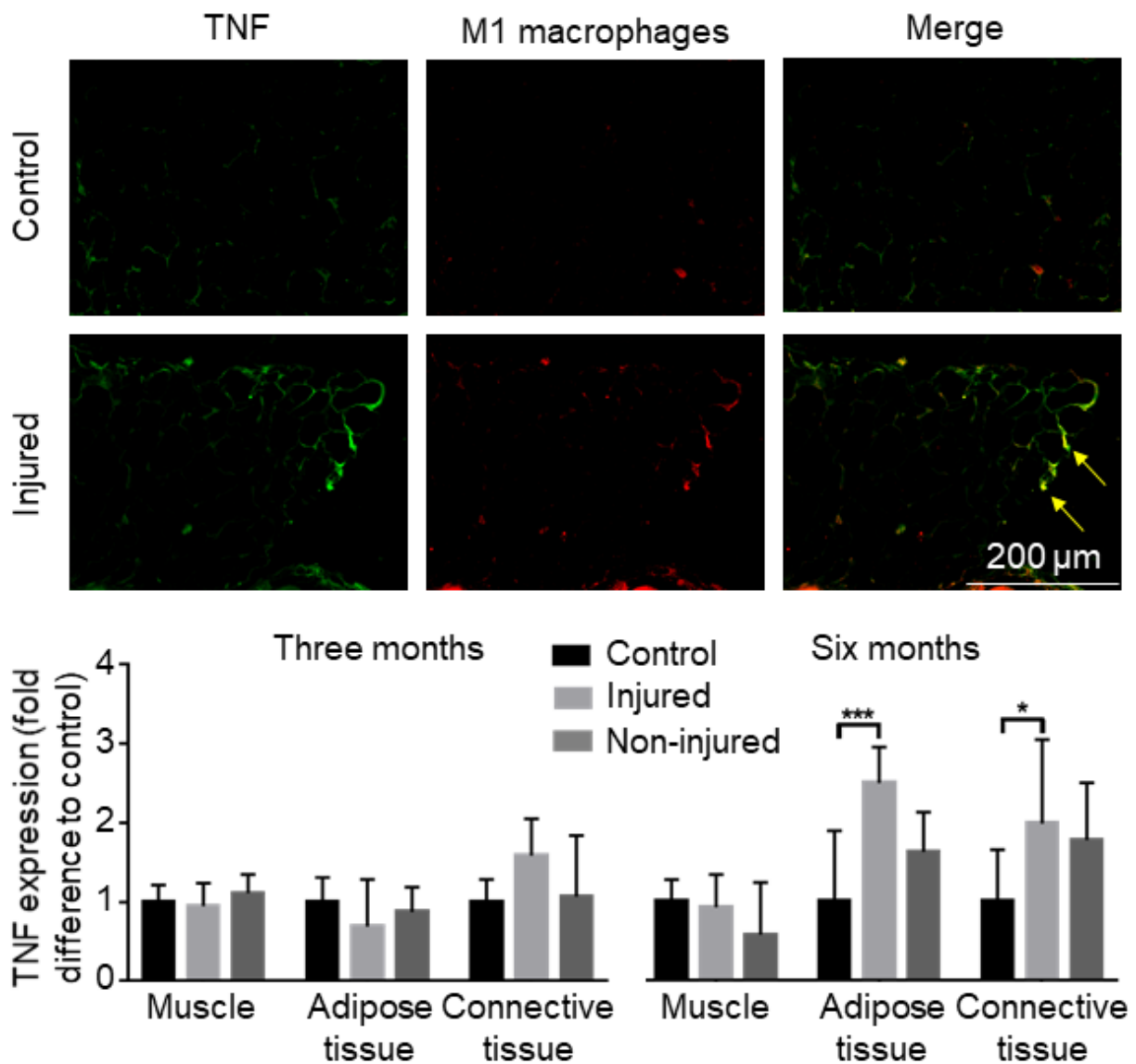
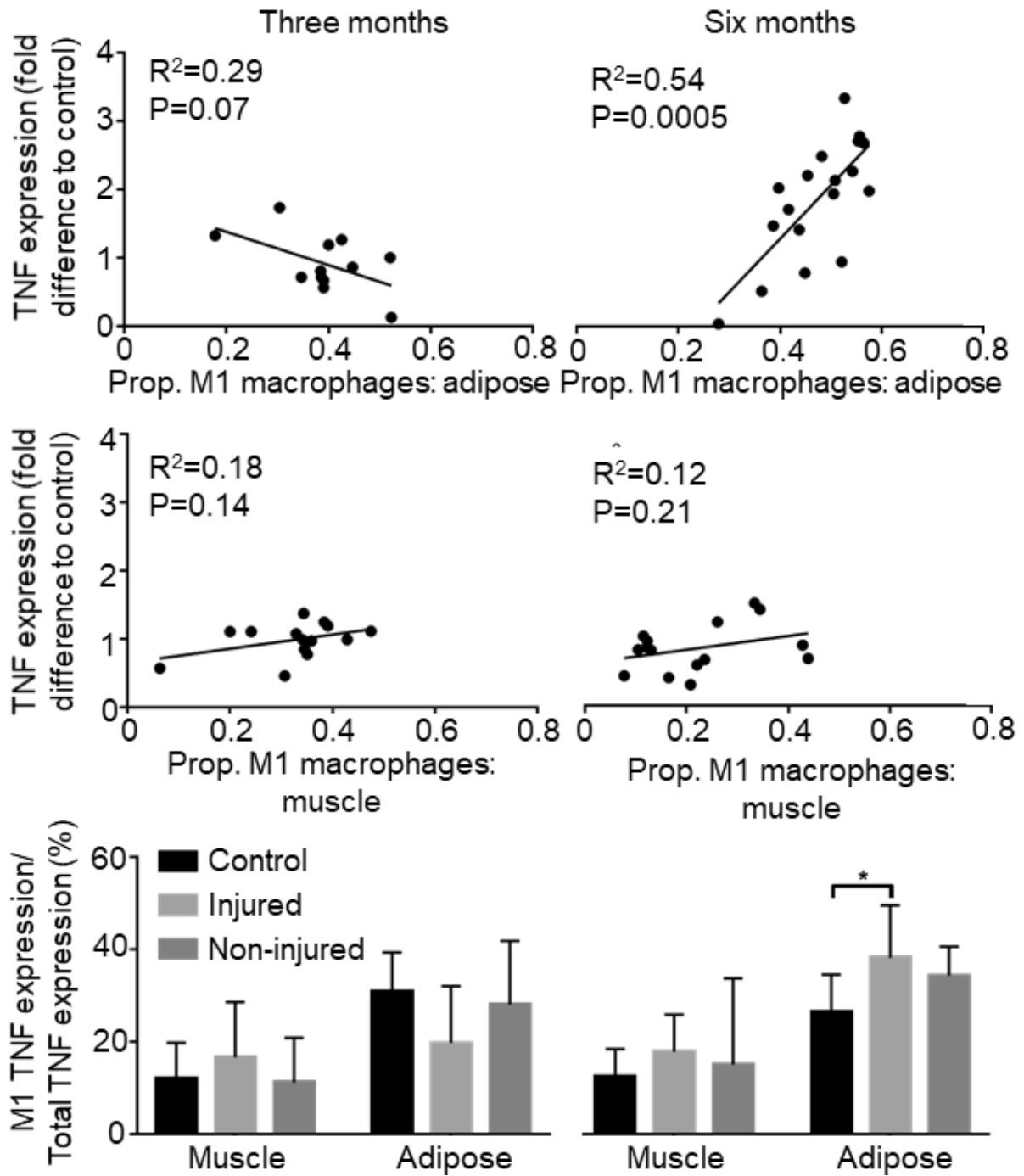


Fig. 7



**Fig. 8**

