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Authors

Davies, Michael R
Lee, Lawrence
Feeley, Brian T
et al.

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Lysophosphatidic Acid-Induced RhoA Signaling and Prolonged Macrophage Infiltration Worsens Fibrosis and Fatty Infiltration Following Rotator Cuff Tears

Michael R. Davies[1,2], **Lawrence Lee**[1], **Brian T. Feeley**[1,2], **Hubert T. Kim**[1,2], and **Xuhui Liu**[1,2]

[1]San Francisco Veterans Affairs Health Care System, Department of Veterans Affairs, 1700 Owens Street, Room 364 San Francisco, California 94153

[2]Department of Orthopaedic Surgery, University of California, San Francisco, California

Abstract

Previous studies have suggested that macrophage-mediated chronic inflammation is involved in the development of rotator cuff muscle atrophy and degeneration following massive tendon tears. Increased RhoA signaling has been reported in chronic muscle degeneration, such as muscular dystrophy. However, the role of RhoA signaling in macrophage infiltration and rotator muscle degeneration remains unknown. Using a previously established rat model of massive rotator cuff tears, we found RhoA signaling is upregulated in rotator cuff muscle following a massive tendon-nerve injury. This increase in RhoA expression is greatly potentiated by the administration of a potent RhoA activator, lysophosphatidic acid (LPA), and is accompanied by increased TNFα and TGF-β1 expression in rotator cuff muscle. Boosting RhoA signaling with LPA significantly worsened rotator cuff muscle atrophy, fibrosis, and fatty infiltration, accompanied with massive monocytic infiltration of rotator cuff muscles. Co-staining of RhoA and the tissue macrophage marker CD68 showed that CD68+ tissue macrophages are the dominant cell source of increased RhoA signaling in rotator cuff muscles after tendon tears. Taken together, our findings suggest that LPA-mediated RhoA signaling in injured muscle worsens the outcomes of atrophy, fibrosis, and fatty infiltration by increasing macrophage infiltraion in rotator cuff muscle. Clinically, inhibiting RhoA signaling may represent a future direction for developing new treatments to improve muscle quality following massive rotator cuff tears.

Keywords

rotator cuff tear; muscle; LPA; macrophage; atrophy; fatty infiltration

Rotator cuff (RC) tears represent a growing disease burden for our aging population, with a full-thickness tear prevalence of 22% in patients 65 and older.[1] While small tears respond well to surgical repair, large tears often have poor outcomes marked by failed repair,

Correspondence to: Xuhui Liu (T: +415-575-0546; F: 415-750-2181; Liux@orthosurg.ucsf.edu).

AUTHORS' CONTRIBUTIONS

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persistent pain, and retear. RC muscle quality is an important predictor of RC repair outcomes, with atrophy and fatty infiltration identified as independent predictors of poor outcomes.[2] Currently, muscle degeneration after massive RC tears is thought to be largely irreversible.

Chronic inflammation has been identified as an important factor responsible for muscle atrophy and degeneration in muscular dystrophy,[3] chronic obstructive pulmonary disease (COPD),[4] cachexia, and sarcopenia.[5] Recently, Gumucio et al. showed increased inflammatory cytokines are accompanied by accretion of macrophages in areas of fat accumulation in RC muscles in a rat RC tear model.[6] These data suggest that macrophage-mediated chronic inflammation is also involved in the development of RC muscle atrophy and degeneration.

Lysophosphatidic acid (LPA) is a small biologically active phospholipid derivative, which binds to its G-protein-coupled receptors, LPAR1, 2, and 3, and leads to increased cell division, migration, and invasion.[7–9] Through these effects on cells, LPA plays diverse roles in the normal development of bone[10] as well as the nervous system.[11] A previous study demonstrated that LPA is a major serum noncytokine survival factor for macrophages.[12] Endogenous LPA has also been implicated in the development of pulmonary[13] and renal fibrosis.[14] A recent study showed that LPA-induced renal fibrosis was mediated through LPA2 receptors and Gαq proteins, resulting in transactivation of latent TGF-β in a Rho/Rho-kinase (ROCK)-dependent manner,[15] suggesting a potential role for both TGF-β and RhoA signaling in the downstream effects of LPA.

RhoA is a member of the small GTPase-Rho family, which also includes Rho, Rac and Cdc42.[16] LPA is an important activator of RhoA[17] through Akt/IκBα signaling.[18] The RhoA signaling pathway is regulated via G protein-coupled receptors and is activated by the interaction between the receptors and ECM and/or soluble factors.[16,19] RhoA activates RhoA kinase (ROCK) to increase turnover of the actin cytoskeleton and has been shown to contribute to monocyte transendothelial migration into tissue.[20] Increased RhoA signaling has been reported in chronic muscle degeneration, such as muscular dystrophy.[21] However, the role of RhoA signaling in RC muscle atrophy and degeneration remains unknown.

The goal of this study was to investigate LPA-induced RhoA signaling in RC muscle degeneration in a rat model. We hypothesized that persistent upregulation of RhoA signaling will lead to a chronic inflammatory state with enhanced macrophage infiltration, leading to increased muscle atrophy, fibrosis, and fatty infiltration.

MATERIALS AND METHODS

Animal Surgery

Sixteen adult female Sprague–Dawley rats underwent complete supraspinatus and infraspinatus tendon transection (TT) with a 5 mm ipsilateral suprascapular nerve resection (DN), as previously described.[22,23] Sham surgery was performed on the contralateral side of each animal. Our previous power analyses determined that N=4 animals/group is sufficient to detect a significant change in muscle weight and protein expression (α=0.05, β 0.2).[22,24]

Thus, we employed four animals per group per time point in this study. All procedures were approved by the San Francisco Veterans Affairs Health Care System (SFVAHCS) Institutional Animal Care and Use Committee (IACUC).

LPA Treatment

Lyosophosphatidic acid was dissolved in 0.1% BSA in PBS and delivered to the animals at a dose of 40 μg/kg via intraperitoneal injection. Injections with dissolved LPA or vehicle (0.1% BSA in PBS) were conducted for 5 out of every 7 days per week in a volume of 0.6 ml for either 2 or 6 weeks. The first dose was given immediately after surgery.

Muscle Harvest

Animals were sacrificed 2 or 6 weeks after surgery. Supraspinatus and infraspinatus muscles were harvested. Supraspinatus muscles were weighed and flash frozen in liquid nitrogen-cooled isopentane for histological processing, as previously described.[24] Infraspinatus muscles were homogenized in Trizol reagent for RNA extraction.

Oil Red-O and Trichrome Staining

Sections were obtained from the muscle belly, away from the musculotendinous junction, at −20°C with a thickness of 10 μm. Total extracellular matrix collagen was assessed using Masson's trichrome staining (Fisher Scientific). Fatty infiltration was assessed with oil red-O staining. The amount of collagen or fat per slide was quantified with Adobe Photoshop as previously described.[25,26] Fibrosis and fat indices were calculated using the equation: Index (%)=(100* (Total area of collagen or fat)/(Total area of the section)). Four serial sections from the muscle belly from each of four animals were used to calculate the indices above.

α-Naphthyl Acetate Esterase and Naphthol AS-D Chloroacetate Esterase Staining

A double esterase staining kit (Sigma–Aldrich, St. Louis, MO) for naphthol AS-D chloroacetate esterase and α-napthyl acetate esterase was used to identify granulocyte-and monocyte-derived cells, respectively, according to manufacturer instructions. Granulocytes were identified as red in color, while cells of the monocytic lineage stained black. Esterase positive cells were counted for four whole muscle sections from each animal (N=4/time point/group). Granulocyte-and monocyte-derived cell density was calculated as number of cells/mm^2 muscle section.

Immunohistofluorescence

CD68 and RhoA co-staining was performed on tissue sections to determine RhoA activity in macrophages. Sections were incubated overnight in rabbit anti-rat RhoA antibody (1:100 dilution, Sigma–Aldrich) with mouse anti-rat CD68 (1:50 dilution, Thermo Scientific, Middletown, VA) and then incubated with goat anti-rabbit Alexa Fluor® 488 and donkey anti-mouse Alexa Fluor® 568-conjugated secondary antibodies at a dilution of 1:5,000 and mounted with VectaShield® Antifade Mounting Medium with DAPI. Slides were visualized with Zeiss Axiovert 200 M fluorescent microscope (Zeiss Inc, Germany).

Real-Time Reverse Transcript Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Burlington, ONT, Canada) according to manufacturer instructions. cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Bioscience, Indianapolis, IN). Real-time qRT-PCR was performed to quantify the expression of RhoA, MIF, GM-CSF, IL-1a, IL-6, IL-10, TNFα, and TGF-β1 in muscle samples using a SYBR Green I Master kit (Roche Applied Bioscience) with the primers found in Table 1. Gene expression was normalized to the housekeeping gene, RPS26. Fold change in mRNA expression was calculated by using $\Delta\Delta C_T$ as described previously.[25,26]

Statistical Analysis

Student's two-tailed *t*-test was used for all statistical analysis between LPA and vehicle treatment groups. Significance was defined as $p<0.05$. Data are presented as the mean ±standard error.

RESULTS

RhoA Is Elevated in Injured RC Muscle

At 2 weeks after injury in vehicle-treated rats, we observed significantly increased RhoA staining in injured muscle, compared to minimal staining in sham-side muscle (Fig. 1). Expression of RhoA was determined to be upregulated fourfold compared to sham at 2 weeks ($p<0.05$) and 1.5-fold at 6 weeks after injury (n.s.) (Fig. 2).

LPA Increases RhoA and Chronic Inflammatory Marker mRNA Expression in Injured Muscle

We found that at both 2 and 6 weeks after injury, total RhoA mRNA levels were significantly elevated in the injured muscle of mice treated with LPA compared to vehicle (Fig. 3). No change in RhoA expression between uninjured sides was observed at either time point.

We next assessed a panel of acute and chronic inflammatory markers at 2 and 6 weeks and found that TNFα was expressed at higher levels in injured muscle of LPA-treated rats at 6 weeks along with a near-significant increase in TGF-β1 expression ($p=0.06$) (Fig. 4). Other inflammatory cytokines, including IL-1a, IL-6, and IL-10 did not show significant differences between LPA and vehicle groups at either time point. The markers of M1 macrophage polarization, MIF, and GM-CSF, likewise did not show significant differences in expression at either time point. We did not observe any differences in the expression of inflammatory markers in the uninjured muscle.

LPA Increases Inflammatory Cells Within RC Muscles

In order to assess the general inflammatory milieu in RC muscles, we performed α-naphtyl acetate esterase and naphthol AS-D-chloroacetate esterase double staining for monocytic and granulocytic cells. At 2 weeks, we observed a significant spike in the number of tissue macrophages present in injured muscle of rats treated with LPA compared to those that received vehicle (Fig. 5A and C). In LPA-treated, injured muscle, the number of α-naphthyl

acetate esterase positive cells outnumbered those staining positive for naphthol AS-D chloroacetate esterase, contrary to what was observed in the injured muscle of rats treated with vehicle. There was no significant difference in the number of naphthol AS-D chloroacetate esterase-positive granulocytes present at 2 weeks in injured muscle between LPA and vehicle groups. At 6 weeks, the number of inflammatory cells present of both the granulocytic and monocytic lineages was increased in the injured muscle of rats that received LPA compared to vehicle, though the numbers of all inflammatory cell types had decreased significantly compared to 2 weeks (Fig. 5B and D).

RhoA Expression Co-Localizes With CD68+ Macrophages in Injured Muscle

Given the high number of monocyte-derived inflammatory cells present in injured RC muscles, we performed immunohistofluorescence to assess the expression patterns of CD68, a macrophage marker, as well as RhoA. We found that at 2 weeks after injury, CD68+ cells strongly co-expressed RhoA at much higher levels than background muscle tissue in both vehicle and LPA-treated tissue (Fig. 6). We noted minimal CD68 or RhoA staining in the uninjured muscle of either group.

LPA Treatment Worsens Muscle Atrophy, Fibrosis, and Fatty Infiltration After Injury

We assessed the gross morphological characteristics of muscles at 6 weeks after injury, at which time point we have previously observed the development of significant muscle atrophy and fibrosis, and early fatty infiltration. At 6 weeks after TT+DN injury, rats treated with LPA demonstrated significantly lower supraspinatus muscle weights on the injury side than those that received vehicle (Fig. 7). This trend was also noted on the sham-side muscle, though was not found to be statistically significant (p=0.08).

We next performed Masson's trichrome staining to determine the fibrosis index of injured muscle, and found that LPA significantly worsens fibrosis of injured muscle with an index of 31.3±5.5% compared to 18.8±3.9% in rats that received vehicle (Fig. 8). Oil red-O staining revealed significantly increased fatty infiltration in the injured muscle of rats that received LPA, with a fat index of 6.3±0.7% compared to 2.6±0.7% in rats that received vehicle (Fig. 9). We observed minimal collagen deposition and no fat in the sham-side muscle of both groups.

DISCUSSION

In this study, we have demonstrated that administration of LPA led to increased RhoA signaling and macrophage infiltration in RC muscles after massive RC tears in our rat model. We further showed that LPA administration led to accelerated RC muscle atrophy, fibrosis, and fatty infiltration. This process was associated with an increase in RhoA expression that co-localized with CD68+ macrophages in injured tissue, suggesting that increased RhoA expression may play a key role in inflammatory macrophage infiltration that occurs after a massive RC tear.

Previous studies of the RhoA pathway in injured muscle have suggested that RhoA inhibition with small molecule inhibitors such as Y-27632 leads to improved histologic outcomes compared to vehicle.[20] Our current study contributes further to the literature by

demonstrating that LPA-induced inflammation and increased RhoA expression worsen muscle atrophy, fibrosis, and fatty infiltration following massive RC tears in rats.

Although RhoA is significantly increased with LPA in RC muscles after injury, we did not observe a strong difference in expression of inflammatory markers between treatment and vehicle groups, including MIF and GM-CSF, markers of M1 macrophage polarization[27] at an early stage (2 weeks) after tendon tears. However, at a later stage (by week 6) after tendon injury, we observed an increase in the expression of the inflammation marker, TNFα. TNFα has been proposed as an intermediate signal in the activation of RhoA by LPA,[28] and has been shown to upregulate RhoA expression via NF-κB signaling in smooth muscle.[29] Additionally, TNFα is elevated in human RC muscle samples of patients with RC tendinopathy and inhibition of TNFα in rats has been shown to improve tendon-to-bone healing following RC tears in rats.[30] That LPA further upregulates TNFα signaling in injured rat muscle compared to vehicle suggests that it accentuates a chronic inflammatory environment analogous to that seen with human RC tears. The near-significant increase in TGF-β1 expression seen with LPA treatment may be related to the increase in monocyte-derived inflammatory cells present in injured muscle. Macrophages are thought to be a key source of TGF-β1 in chronically injured muscle,[31] and TGF-β1 signaling has been linked to increased fibrosis within the RC of rats and increased fibrofatty infiltration in mice.[23,32] Inhibition of TGF-β1 signaling in mice has likewise shown a reduction in RC muscle atrophy and fibrofatty infiltration by decreasing the number of fibro/adipogenic progenitor cells present in injured muscle.[32] Interestingly, a recent study showed that LPA-induced renal fibrosis was mediated through LPA2 receptors and Gαq proteins via transactivation of TGF-β in a Rho/Rho-kinase (ROCK)-dependent manner, suggesting another possible connection between LPA signaling and a pathway previously linked to muscle degeneration.[15] Taken together, this suggests that LPA-induced TGF-β expression may be one mechanism by which we observed increases in fibrosis and fatty infiltration compared to vehicle in this study.

We next sought to characterize the pattern of inflammatory cells present in injured RC muscle at early (2 weeks) and later (6 week) stages after RC injury. We were surprised to find a significant increase in the number of monocyte-derived, α-naphthyl acetate esterase-positive cells at 2 weeks after injury in rats treated with LPA compared to those seen in rats that received vehicle. This difference in inflammatory cell number in injured muscle between LPA and vehicle groups persisted at 6 weeks, although inflammatory cells were globally decreased at this later time point compared to 2 weeks. Having observed a striking increase in α-naphthyl acetate esterase-positive cells at 2 weeks in the LPA treatment group compared to vehicle, we next characterized the expression pattern of CD68, a surface marker of tissue macrophages, in the context of the elevated RhoA expression seen with LPA treatment. We found that CD68+ cells in injured muscle strongly co-expressed RhoA in nearly all cases observed. This pattern was not observed in uninjured muscle, in which there was little expression of CD68 or RhoA. A previous study noted co-localization of CD68+ microglia and RhoA in the setting of multiple sclerosis,[33] but this is the first study, to our knowledge, to describe this finding in the setting of a tendon-nerve injury in muscle. Worthylake et al. have shown that inhibiting RhoA within monocytes inhibits their ability to undergo transendothelial migration,[34] preventing them from maturing into tissue

macrophages. We postulate that activated monocytes and macrophages express RhoA in response to an injury stimulus, and that LPA, by increasing activation and expression levels of RhoA, potentiates the migration and polarization of macrophages in response to injury, leading to a greater number of tissue macrophages in injured muscle. In chronic muscle injury, this likely leads to degenerative changes including fibrosis and fatty infiltration and thus is a potential future pharmacologic target to improve muscle function after RC repair.

This study has certain inherent limitations. First, our sample size is relatively small, though sufficient to detect statistically significant differences between groups in this study. Second, only two time points were adopted in this study. Inflammation is a highly dynamic process that cannot be fully analyzed from two time points alone, and future studies would benefit from the addition of time points earlier than 2 weeks as well as time points later than 6 weeks. In spite of this, we are still able to draw strong conclusions about the impact of LPA and chronic inflammation on injured muscle from the time points observed in this study. Additionally, we did not perform inhibition of RhoA signaling in this study, though this may be the focus of future experiments. Differentiating the fraction of GTP-bound, active RhoA from total RhoA levels was also beyond the scope of this study, but is of interest for future experiments. Finally, we did not quantify the number of CD68+ positive cells that co-expressed RhoA on histology, though co-expression was observed for nearly all CD68+ cells in both the LPA and vehicle groups.

We have observed a marked connection between muscle inflammation, RhoA+ macrophage infiltration, and worsened muscle atrophy, fibrosis, and fatty infiltration in a pre-clinical model of massive RC tear. Our findings suggest that LPA-mediated inflammation worsens RC muscle degeneration in rats, and thus further characterizing the role of RhoA and other mediators of inflammation following RC injury is of great clinical and therapeutic importance.

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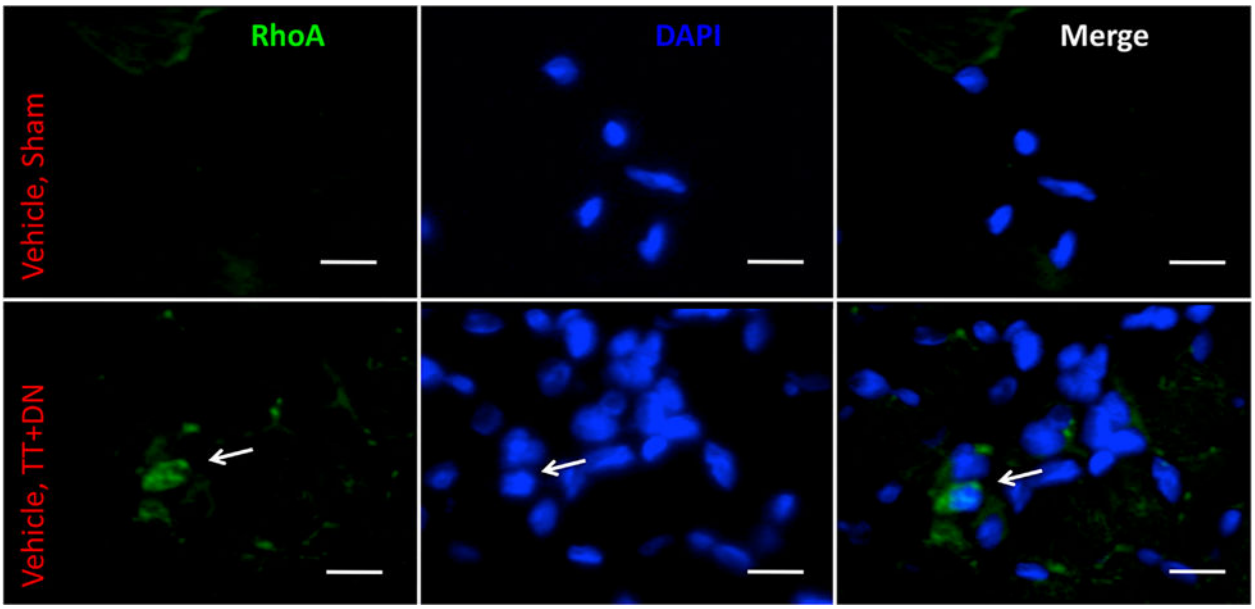

Figure 1.

Representative immunohistofluorescence demonstrating increase in total RhoA expression in injured muscle (bottom row) compared to uninjured muscle (top row). White arrow indicates RhoA-positive cell. Scale bar=10 μm.

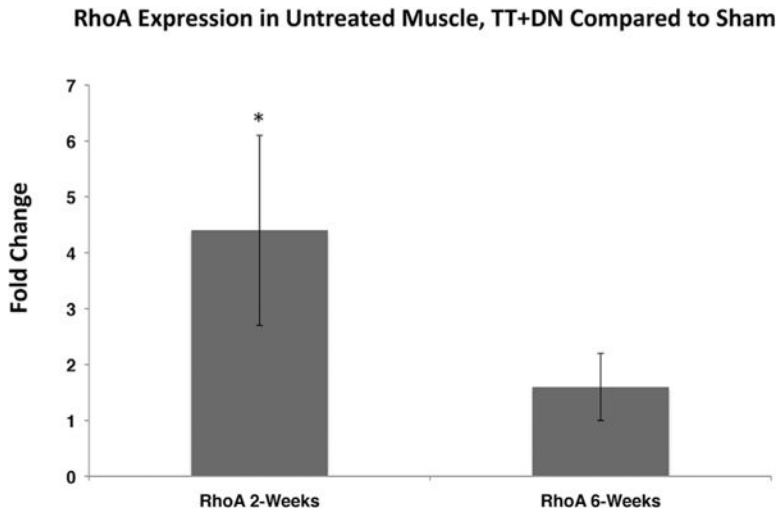

Figure 2.

Real-time qRT-PCR demonstrating increase in total RhoA expression at 2 and 6 weeks after injury and initiation of treatment, TT+DN ΔC_T values normalized to mean sham ΔC_T values to calculate $\Delta\Delta C_T$. Student's two-tailed *t*-test was performed between ΔC_T values of injured and sham muscle, $*p<0.05$.

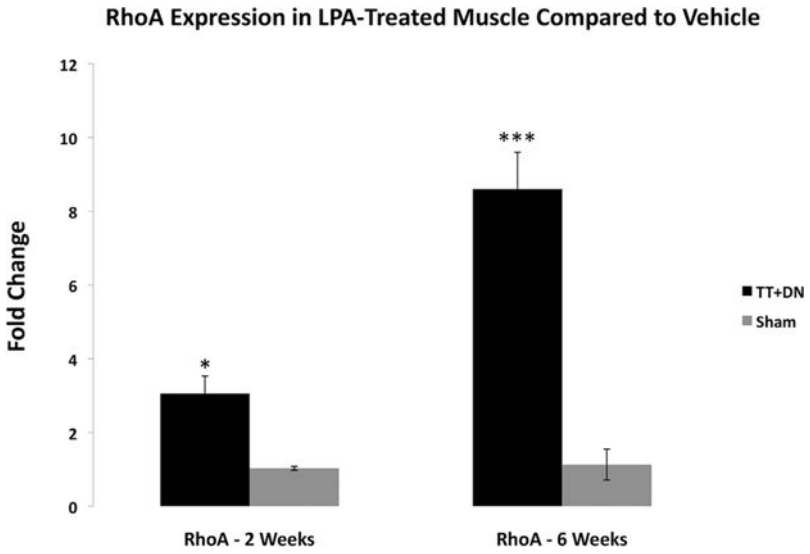

Figure 3.

Real-time qRT-PCR comparing the effects of LPA vs. vehicle on total RhoA expression at 2 and 6 weeks after injury and initiation of treatment, LPA-treatment ΔC_T values normalized to those for the vehicle group for both TT+DN and sham muscle to calculate $\Delta\Delta C_T$. Student's two-tailed *t*-test was performed between ΔC_T values of LPA treatment and vehicle groups for both injured and sham muscle, $*p<0.05$, $***p<0.001$.

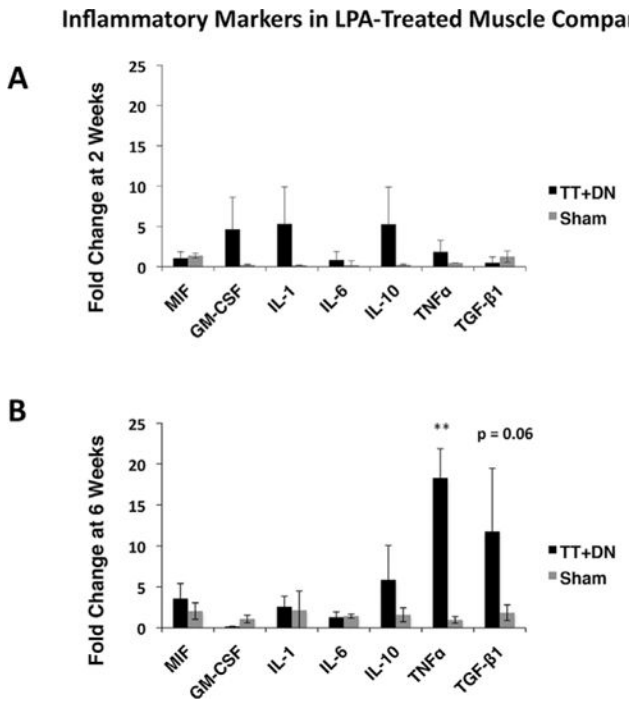

Figure 4.

Real-time qRT-PCR comparing the effects of LPA versus vehicle on inflammatory markers at 2 and 6 weeks after injury and initiation of treatment, LPA-treatment ΔCT values normalized to those for the vehicle group for both TT DN and sham muscle to calculate $\Delta\Delta C_T$. Student's two-tailed *t*-test was performed between ΔC_T values of LPA treatment and vehicle groups for both injured and sham muscle, $*p<0.01$.

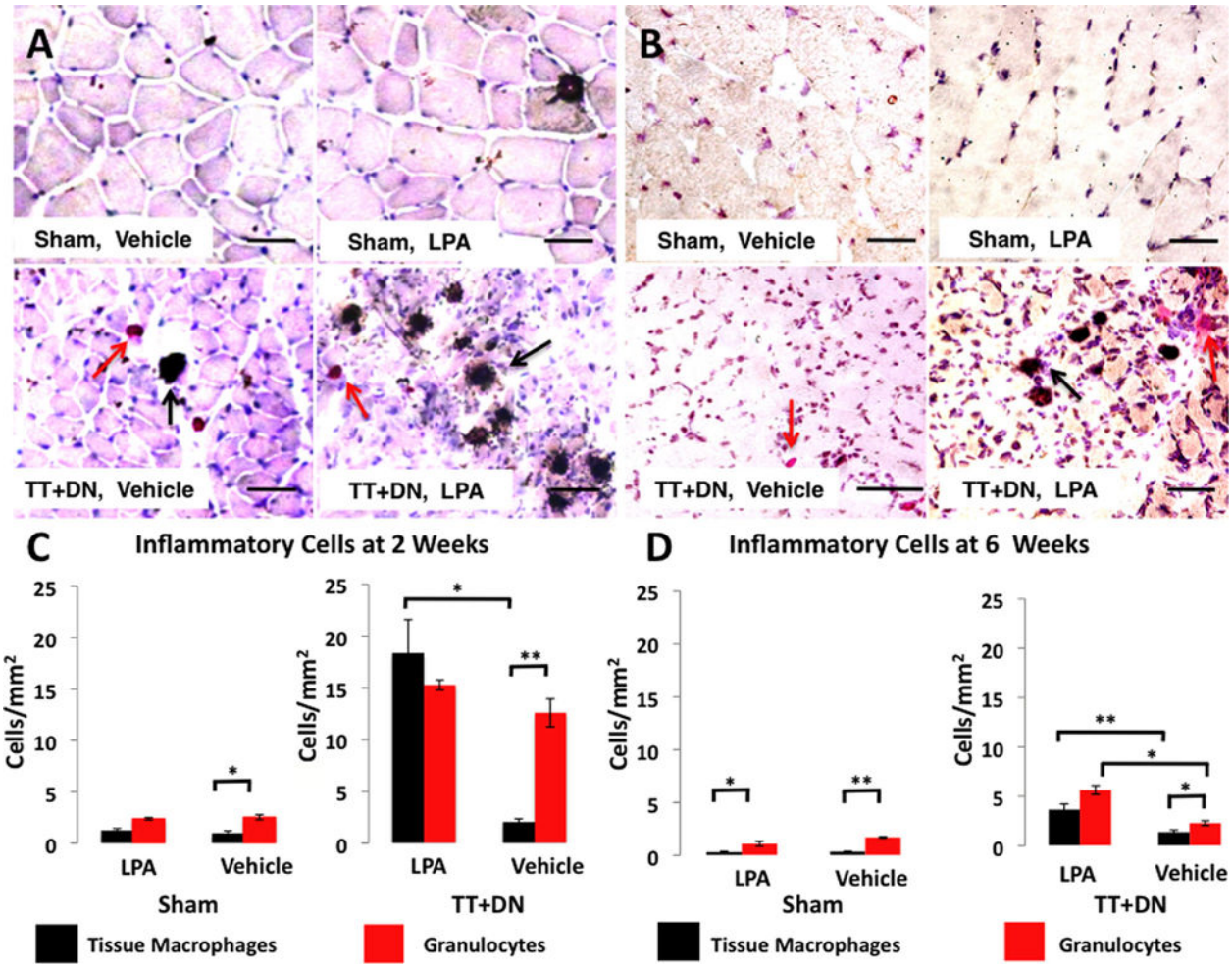

Figure 5.

Representative double-esterase staining at 2 weeks (A and C) and 6 weeks (B and D) after injury. Black arrows indicate α-naphthyl acetate esterase positive, monocyte-derived cells; red arrows indicate naphthol ASD-chloroacetate esterase positive, granulocyte-derived cells. Scale bar=50 μm. Cell density shown in C–D, *$p<0.05$, *$p<0.01$.

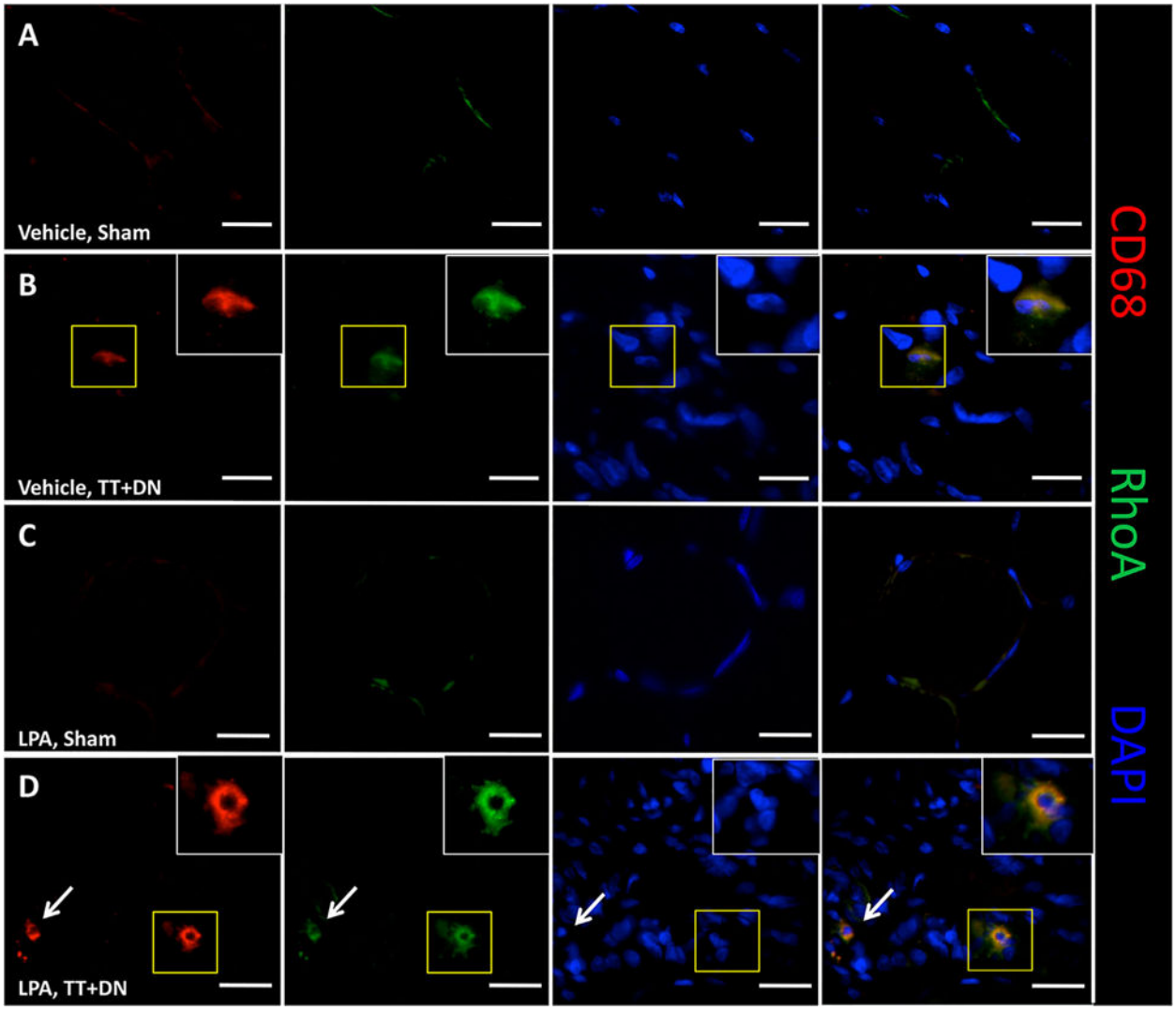

Figure 6.

Representative immunohistofluorescence showing co-expression of CD68 and RhoA in tissue macrophages in rotator cuff muscles. Area within yellow box is enlarged in white box. Scale bar=20μm. White arrow (bottom row) indicates an additional CD68+/RhoA+ cell.

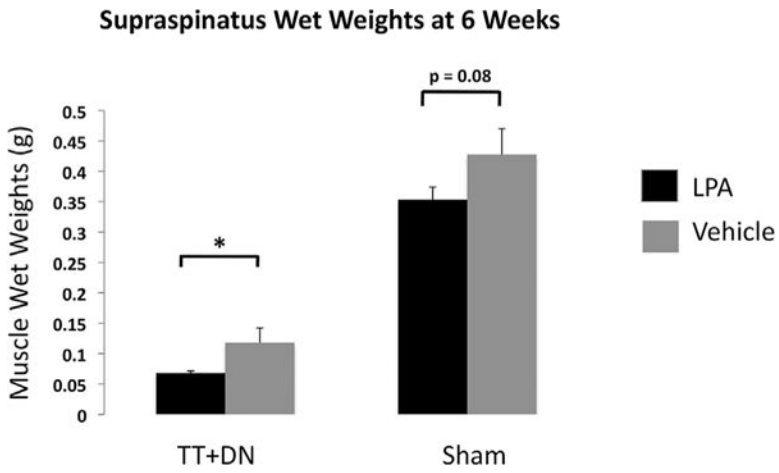

Figure 7.

LPA treatment significantly reduced muscle wet weights (g) at 6 weeks after TT+DN injury, but not after sham surgery.

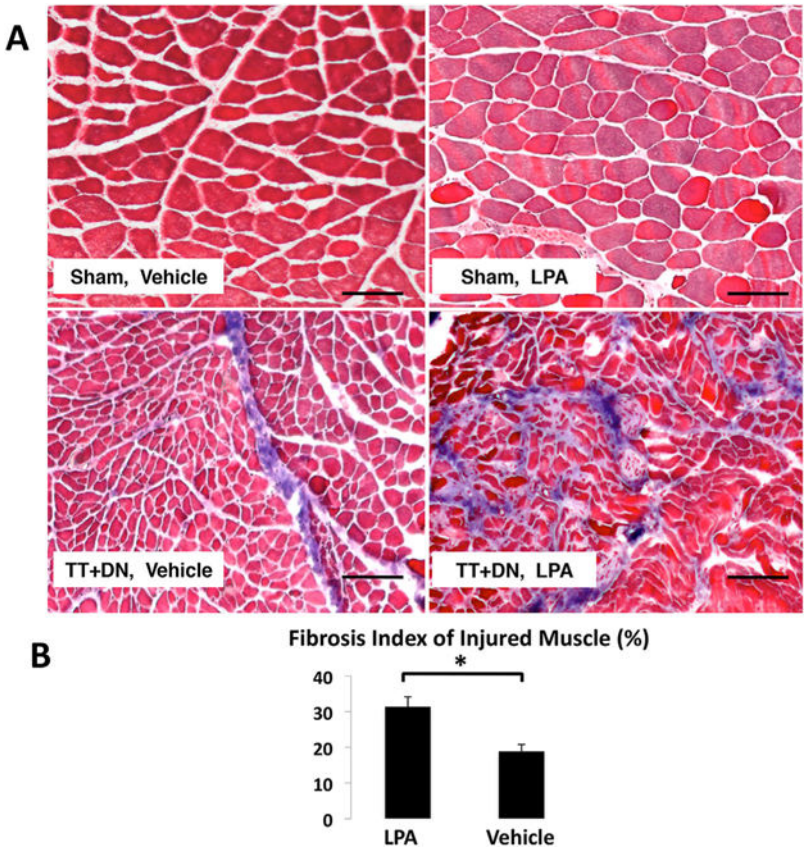

Figure 8.

(A) Representative Masson's trichrome stain at 6 weeks after injury (Scale bar=100 μm.). (A) Quantitative analysis of fibrosis index showed LPA treatment significantly increased fibrosis of rotator cuff muscles after TT DN injury (*p<0.05).

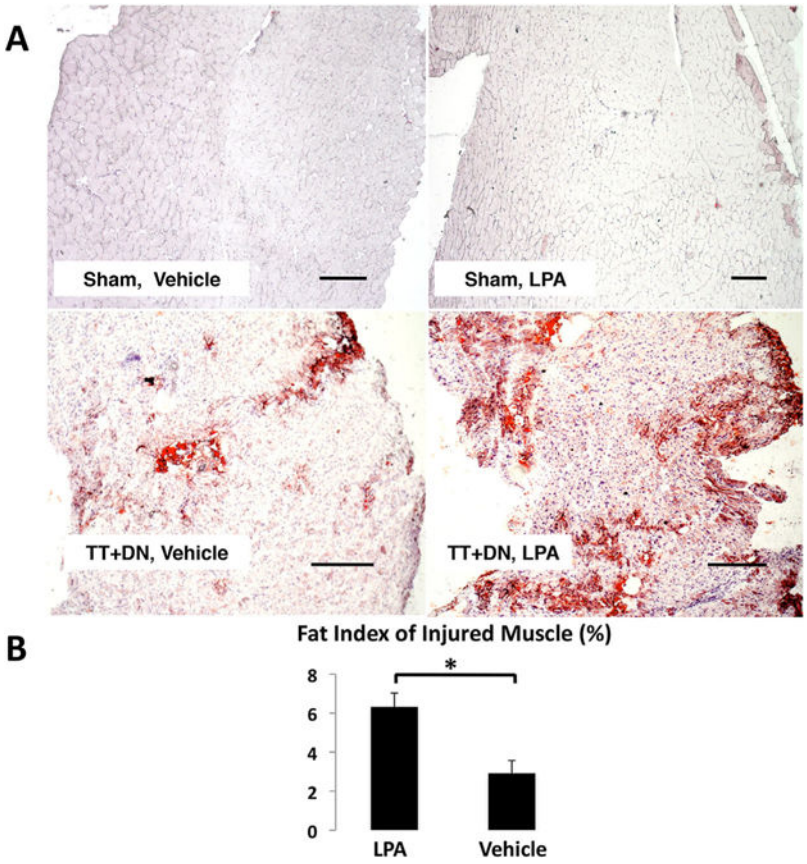

Figure 9.

(A) Representative oil red-O stain at 6 weeks after injury (Scale bar=500 μm). (B) Quantitative analysis of fatty infiltration index showed LPA treatment significantly increased fatty infiltration of rotator cuff muscles after TT DN injury (*p<0.05).

Table 1

Sequences of Primers Used for Real-time qRT-PCR

Primer	Forward (5′ → 3′)	Reverse (5′ → 3′)
RhoA	GTAGAGTTGGCTTTATGGGACAC	TGFAGTCCATTTTTCTGGGATG
TGF-β1	GGAGCCACTGCCCATCGTCTACTAC	GGAGCGCACGATCATGTTGGAC
IL-1a	AAGACAAGCCTGTGTTGCTGAAGG	TCCCAGAAGAAAATGAGGTCGGTC
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
IL-10	CAATAACTGCACCCACTTCC	ATTCTTCACCTGCTCCACTGC
TNFα	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
GMCSF	TAAATGACATGCGTGCTCTGG	ATGAAATCCTCAAAGGTGGTG
MIF	CCATGCCTATGTTCATCGTG	GAACAGCGGTGCAGGTAAGTG
RPS26	AAGGAGAAACAACGGTCGTG	GCAGGTCTGAATCGTGGTG