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Quantitative analysis of immune cell subset infiltration of supraspinatus muscle after severe rotator cuff injury

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Abstract

Rotator cuff tears cause muscle degeneration that is characterized by myofiber atrophy, fatty infiltration, and fibrosis and is minimally responsive to current treatment options. The underlying pathogenesis of rotator cuff muscle degeneration remains to be elucidated, and increasing evidence implicates immune cell infiltration as a significant factor. Because immune cells are comprised of highly heterogeneous subpopulations that exert divergent effects on injured tissue, understanding trafficking and accumulation of immune subpopulations may hold the key to more effective therapies. The present study quantifies subpopulations of immune cells infiltrating the murine supraspinatus muscle after severe rotator cuff injury that includes tenotomy and denervation. Rotator cuff injury stimulates dramatic infiltration of mononuclear phagocytes, enriches mononuclear phagocytes in non-classical subpopulations, and enriches T lymphocytes in T_H and T_{reg} subpopulations. The combination of tenotomy plus denervation significantly increases mononuclear phagocyte infiltration, enriches macrophages in the non-classical subpopulation, and decreases T lymphocyte enrichment in T_H cells compared to tenotomy alone. Depletion of circulating monocytes via liposomal clodronate accelerates supraspinatus atrophy after tenotomy and denervation. The study may aid rational design of immunologically smart therapies that harness immune cells to enhance outcomes after rotator cuff tears.

Introduction

Full-thickness rotator cuff tears (RCTs) are present in more than 20% of the population[1]. RCTs increase in frequency and severity with age and cause significant pain and functional deficiency[2]–[6]. RCT causes degeneration of the associated muscles, a process which includes muscle retraction and atrophy, fatty infiltration, and fibrosis[5], [7], [8]. Muscle degeneration is a strong predictor of patient morbidities such as pain, functional deficiency, and post-surgical tear recurrence, and is not reversed by tendon repair[9], [10]. Thus, prevention or reversal of muscle degeneration due to RCT is a major unmet clinical need. Identification of molecular and cellular targets for therapeutic intervention requires elucidating the underlying pathobiology of RC muscle degeneration.

Evidence from RCT and chronic muscle pathologies suggests that inflammation, particularly mononuclear phagocyte (MP) infiltration, contributes to muscle degeneration. Pro-inflammatory cytokines such as TNF α and IL-6 stimulate apoptosis of myocytes and catabolism of intramyocellular proteins [11], [12], thus causing muscle atrophy in cancer cachexia and autoimmune disorders. Classical “pro-inflammatory” subpopulations of mononuclear phagocytes secrete more pro-inflammatory cytokines compared to non-classical alternatively-activated subpopulations [13]–[16], suggesting that intramuscular infiltration of classical subtypes may promote chronic muscle degeneration. Indeed, in the *mdx* mouse model of Duchenne muscular dystrophy, chronic muscle degeneration is in part caused by the classical pro-inflammatory Ly6C^{hi} subset of circulating monocytes (MO) [17]. Human and rodent muscles undergoing fatty degeneration after RCT show dramatic co-localization of fat-rich regions with macrophages (M Φ) that contain intracellular lipid droplets [7], [8]. Rotator cuff muscle degeneration is exacerbated by administration of lysophosphatidic acid, whereby M Φ accumulation and TNF α expression are increased [18]. Taken together, these studies suggest that pro-inflammatory mononuclear phagocytes promote chronic muscle degeneration.

Because MP are comprised of highly heterogeneous subpopulations that exert divergent effects on injured tissue during pathogenesis and regeneration, understanding RC muscle inflammation at the subpopulation level is critical for identifying therapeutic targets. The spectrum of M Φ phenotypes is commonly simplified into two primary categories: classically-activated “M1” and non-classical or alternatively-activated “M2”, of which multiple subtypes have been described [14]. Accumulation of M Φ within injured adult skeletal muscle is primarily driven by recruitment of circulating MO rather than expansion of tissue-resident M Φ [19]. MO circulate as functionally distinct subsets in both mouse and human blood. Classical MO are characterized by Ly6C^{hi}CX3CR1^{lo} expression in mice (CD14^{hi}CD16⁻ in human), whereas non-classical MO are Ly6C^{lo}CX3CR1^{hi} in mice (CD14⁺CD16⁺ in human) [20]. Ly6C^{hi} classical MO predominate the acute phases of injury, secrete inflammatory cytokines such as IL-6, iNOS, and TNF α [21], and produce high levels of matrix metalloproteinases and cathepsins [16], whereas Ly6C^{lo} MO predominate in later phase inflammation, promote angiogenesis and matrix deposition, and secrete higher levels of VEGF, TGF β , and IL-10 and lower levels of TNF α and IL-1 β [16], [19], [22]. MO recruitment is required for natural repair; multiple studies show that depletion of circulating MO causes incomplete and fibrotic healing of skeletal muscle after toxin-induced injury [19], [23], [24]. In early stages after acute muscle injury, infiltrating Ly6C^{hi} MO ingest debris and induce proliferation of myosatellite mononuclear progenitor (satellite) cells [19]. In later stages, Ly6C^{lo} MO and M2 M Φ subsets promote satellite cell myogenic differentiation and fusion to expand myotubes, and secrete insulin-like growth factor-1 (IGF-1) required for muscle regeneration [25].

Given that different subpopulations of MP exert distinct effects on skeletal muscle, the present study seeks to investigate the phenotypic distribution and causal role of inflammatory cells in supraspinatus (SS) degeneration after severe RCT in a mouse model.

Results

To quantify subpopulations of inflammatory cells within the SS muscle after massive RC injury, SS muscles were harvested 7 days after injury and analyzed by flow cytometry. MP were identified according to the following surface marker expression: $M\Phi = \text{MerTK}^+ \text{CD64}^+$; dendritic cell (DC) = $\text{NOT}(\text{MerTK}^+ \text{CD64}^+) \text{CD11c}^+$; MO = $\text{NOT}(\text{MerTK}^+ \text{CD64}^+) \text{CD11c}^- \text{CD11b}^+ \text{SSC}^{\text{lo}}$ (Fig 1a)[26]. MP count is 3.9×10^4 cells/g in the tenotomy (TT) group and 8.7×10^4 cells/g in the tenotomy plus denervation (TT+DN) group, which represent 3.2-fold and 3.9-fold increases from respective uninjured contralateral muscles (Fig 1b). MP count is 2.2-fold greater in TT+DN than TT (Fig 1b). $M\Phi$ count is 3.0×10^4 cells/g in the TT+DN group, increasing 6.3-fold compared to contralateral (Fig 1c). MO count is 1.0×10^4 in TT and 2.3×10^4 in TT+DN, representing 2.4-fold and 2.6-fold increases compared to contralateral controls (Fig 1d). MO count is 2.3-fold greater in TT+DN than TT (Fig 1d). DC count in the TT+DN group is 3.9-fold higher than contralateral and 2.1-fold higher than TT (Fig 1e). The frequency of DC, $M\Phi$, and MO populations is 1.7%, 1.2%, and 1.1% in TT, respectively, compared to 2.5%, 2.1%, and 1.6% in TT+DN (Fig 1f). Overall, results indicate that injury causes dramatic accumulation of MO, $M\Phi$, and DC.

M1-like and M2-like subpopulations of $M\Phi$ were identified by surface expression of CCR7 and CD206, respectively (Fig 2a), as per Spiller *et al.*[13]. TT increases the frequency of CD206^+ cells within the $M\Phi$ pool from 29.3% to 59.9%; TT+DN, from 39.8% to 74.7% (Fig 2b). CD206^+ frequency is higher in TT+DN compared to TT (Fig 2b). Conversely, TT decreases the frequency of CCR7^+ cells within the $M\Phi$ pool from 77.7% to 59.6%; TT+DN, from 82.7% to 64.2% (Fig 2c). Thus, SS muscle is biased toward M1-like $M\Phi$ without injury, and toward M2-like $M\Phi$ after TT and TT+DN. The TT+DN group has the highest counts of both CD206^+ $M\Phi$ and CCR7^+ $M\Phi$ compared to TT and contralateral (Fig 2d). Increased CCR7^+ $M\Phi$ count despite decreased CCR7^+ $M\Phi$ frequency in the $M\Phi$ pool is explained by the larger overall pool of $M\Phi$ in the TT+DN group.

MO subpopulations are discriminated by surface expression of Ly6C (Fig 3a) [16], [20]. TT +DN significantly increases the frequency of non-classical Ly6C^{lo} (Fig 3b) and decreases the frequency of classical Ly6C^{hi} (Fig 3c) cells within the MO pool compared to contralateral, whereas TT has no significant effect on $\text{Ly6C}^{\text{lo}}/\text{Ly6C}^{\text{hi}}$ frequency. Consequently, TT+DN increases the absolute count of both Ly6C^{lo} and Ly6C^{hi} compared to TT and contralateral (Fig 3d). TT increases Ly6C^{lo} count compared to contralateral, owing increased overall MO count (Fig 3d).

Neutrophil and T lymphocyte infiltration is also quantified (Fig 4a). Neutrophils (Ly6G^+) are present in low abundance (6000 cells/g) relative to other inflammatory cell types measured. Neither injury affects neutrophil count (Fig 4b) but TT increases the frequency of neutrophils out of total cells compared to TT+DN (Fig 4c). T lymphocyte count is unaffected by either injury (Fig 4d), but the frequency of T lymphocytes decreases due to TT (Fig 4e). Helper T lymphocyte (T_H ; $\text{CD4}^+\text{CD3}^+$) and regulatory T lymphocyte (T_{reg} ; $\text{CD25}^+\text{CD4}^+\text{CD3}^+$) counts and frequency out of total cells do not change due to injury (Fig 4f, g, i, j). Injury enriches the T lymphocyte pool in T_H cells, as evidenced by TT increasing the T_H frequency within the T lymphocyte pool from 2.8% to 7.5% and TT+DN increasing

the frequency from 3.2% to 4.9% relative to controls (Fig 4h). The T_H frequency within the T lymphocyte pool is higher in TT compared to TT+DN (Fig 4h). Neither injury enriches the T_H pool in T_{reg} cells (Fig 4i). The T_{reg} frequency within the T lymphocyte pool is higher in TT (3.1%) compared to TT+DN and contralateral (Fig 4k), due to the larger frequency of T_H .

To describe the inflammatory profile in a reduced-dimensionality model, unsupervised principal component (PC) analysis was performed on a set of 11 flow cytometric measurements. Injured versus contralateral muscles are discriminated along PC1, and TT versus TT+DN muscles are discriminated along PC2 (Fig 5b). The x-axes of the loadings (Fig 5a) and scores (Fig 5b) plots show that injury is described by increased MO, $M\Phi$, DC, $Ly6C^{lo}$ MO, $CD206^+ M\Phi$, T_H , and T_{reg} , and decreased $CCR7^+ M\Phi$, T lymphocytes, and wet muscle mass. The y-axes of the loadings and scores plots show that TT+DN is discriminated from TT by increased MO, $M\Phi$, DC, $CD206^+ M\Phi$, and T lymphocytes, and decreased T_H , T_{reg} , and neutrophils.

Intravenous administration of liposomal clodronate is an experimental tool used to deplete circulating MO [27], [28]. Liposomal clodronate causes complete depletion of blood MO within 6 h followed by recovery of the $Ly6C^{hi}$ and $Ly6C^{lo}$ populations at 2 and 7 days, respectively [28]. The present study investigates the contribution of circulating MO to SS atrophy by administering liposomal clodronate (clod-lip) or saline control every 2–3 days upon combined tenotomy and denervation injury (Fig 6a). Clod-lip decreases wet muscle mass compared to uninjured contralateral control at 21 days (mean: 29.0 vs. 41.8 mg), whereas saline control has no effect (mean: 38.5 vs. 41.5 mg) (Fig 6b). Clod-lip causes greater percent decrease in wet mass between injured and uninjured contralateral muscles compared to saline control (Fig 6c). Quantification of myofiber cross-sectional area (CSA) is a complementary approach to assess muscle atrophy and is accomplished using immunohistochemistry (IHC) to visualize collagen IV in the myofiber basement membrane (Fig 6d). Saline control shows no difference in myofiber CSA between injured and uninjured contralateral muscles (median: 607 vs. 655 μm^2) (Fig 6e). Lack of atrophy at 21 days in the saline control group is consistent with literature indicating that mouse SS degeneration is detected at 6–12 weeks [29]–[31]. Notably, clod-lip causes decreased CSA compared to both uninjured contralateral (median: 507 vs. 641 μm^2) (Fig 6f) and saline controls (Fig 6g). Taken together, these results indicate that depletion of circulating MO via liposomal clodronate accelerates SS atrophy after severe RC injury.

Discussion

RCT treatment responses are poor, as non-operative management fails in approximately 50% of patients, leaving surgical tendon repair as the standard of care [4], [5]. The failure rate of surgery is as high as 26% for small to medium tears and up to 94% for large and massive tears [32]–[37]. Because muscle degeneration correlates highly with patient morbidities such as pain, functional deficiency, and post-surgical tear recurrence, prevention or reversal of muscle degeneration due to RCT could significantly improve treatment outcomes.

By selectively targeting subpopulations of MP that are present in the injury niche of the torn RC, new interventions may be found that harness endogenous inflammatory signaling to promote repair. Our group and others have previously investigated this therapeutic approach in diverse injury models, including volumetric muscle loss[38], peripheral nerve regeneration[39], bone regeneration[40]–[43], microvascular network growth[44], [45], and cardiovascular injury [46]. Application of immuno-regenerative biomaterial strategies to treat RCT requires a foundational understanding the subpopulations present and their functions in this injury context. The present study represents the first detailed quantification of inflammatory cell populations in SS muscle and their role in degeneration.

RC injury causes dramatic infiltration of MP comprised of comparable frequencies of MΦ, MO, and DC (Fig 1). Injury skews the MΦ and MO pools toward non-classical CD206⁺ and Ly6C^{lo} subpopulations, respectively, and away from the classical CCR7⁺ and Ly6C^{hi} subpopulations (Fig 2, 3). The impact of non-classical MΦ and MO on skeletal muscle has been partially elucidated in both acute and chronic injury contexts. After toxin-induced injury to skeletal muscle, CD206⁺ MΦ and Ly6C^{lo} MO accumulate during the secondary phase of healing and are required for complete regeneration, likely because they stimulate myotube fusion[19]. COX-2^{lo} MΦ, which are phenotypically similar to CD206⁺ MΦ, protect myotubes against atrophy *in vitro* [47]. In contrast, classical Ly6C^{hi} MO promote chronic muscle degeneration in *mdx* mice[48]. Taken together, these findings suggest that non-classical MO and MΦ may support SS integrity compared to their classical counterparts that may feed forward degeneration. Here, depletion of circulating MO via liposomal clodronate accelerates SS atrophy (Fig 6). Because infiltration of circulating MO supplies the intramuscular MP pool[19], which is skewed toward non-classical MO and MΦ after RCT, these results suggest that non-classical MO and MΦ may protect against SS degeneration. Therefore, interventions that decrease MP accumulation may exacerbate outcomes after RCT. Toward the ultimate goal of therapeutic immunomodulation, follow-up studies should employ gain- and loss-of-function techniques to elucidate the functions of non-classical MO and MΦ in the context of SS degeneration and regeneration. Because these typically “pro-regenerative” MP subpopulations predominate the SS as early as day 7, increasing their recruitment may offer only modest therapeutic efficacy for muscle degeneration, which occurs over weeks. A more promising strategy may be to bias recruited cells toward pro-regenerative cytokine secretion using appropriately engineered delivery of immune modulatory molecules[44], [49], [50].

MO and MΦ infiltration of SS muscle is less pronounced after RCT compared to other muscle injury contexts. MO and MΦ counts are 2.3×10^4 and 3.0×10^4 cells/g at day 7 following TT+DN (Fig 1). In degenerative muscles of the *mdx* mouse model of muscular dystrophy, MΦ counts vary from 6×10^4 to 3×10^6 cells/g at 6–12 weeks of age[48], [51]. After toxin-induced skeletal muscle injury, MO/MΦ infiltration is dramatically higher, reaching a maximum of 1.4×10^7 cells/g at day 7[52]. Myocardial infarction results in massive infiltration of 4.5×10^7 Ly6C^{hi} and 3.0×10^7 Ly6C^{lo} MO/g at days 3 and 5–7, respectively[16]. Notably, the preceding examples involve direct injury to the muscle, whereas the RCT model herein primarily involves injury to tendon and nerve, with subsequent effects on rotator cuff muscles. That RCT results in less infiltration of MP

compared to other muscle pathologies suggests that the underlying inflammatory stimuli may be less severe.

The present study represents a snap shot of the immune cell profile, which may change dynamically over time. The day 7 time point was selected because robust MP infiltration is detected at day 7 in numerous studies of soft tissue injury[38], [44], [52]–[54]. Evaluating multiple time points earlier and later than day 7 would elucidate whether the magnitude and phenotypic distribution of MP infiltration evolves temporally after RCT.

The ideal injury paradigm for pre-clinical investigation of RCT is controversial. Specifically, it is unclear whether denervation is an appropriate adjunct injury to tenotomy for modeling human pathogenesis. Clinically, the relationship between tendinopathy, suprascapular neuropathy, and muscle degeneration is unclear. Suprascapular neuropathy is present in only 8–12% of patients with full-thickness RCTs [55]–[59] and is likely caused by muscle retraction and increased tension on the suprascapular nerve [60]. Clinical studies contradict each other as to whether RCT-induced suprascapular neuropathy contributes to muscle degeneration [57], [61]. Nevertheless, in pre-clinical models, suprascapular denervation helps recapitulate the degree of muscle degeneration observed in human cases of RCT[30], [31], [62]. In the present study, TT+DN increases MP, MO, and DC counts by at least 2-fold (Fig 1) and slightly increases CD206⁺ enrichment of the MΦ pool compared to TT (Fig 2). These results show that inflammatory infiltrate in SS muscle is affected dramatically by denervation as an adjunct to tenotomy.

The biological cascade connecting RCT-induced muscle unloading to chronic muscle degeneration is unknown. Given that muscle anabolism and catabolism are coupled to physical activity[63], mechanical unloading after RCT likely causes myofiber atrophy by shifting muscle metabolism from anabolic to catabolic processes. The initial hypothesis of the present study is that MP infiltration exacerbates SS atrophy. According to this hypothesis, muscle unloading after RCT causes mitochondrial dysfunction[64], elaboration of damage associated molecular patterns (DAMPs), and recruitment of inflammatory cells that secrete pro-inflammatory cytokines (e.g. TNFα, IL-1b, IL-6)[14]–[16], [65], which then stimulate catabolism of intramyocellular proteins over weeks[12]. However, results here suggest that MP infiltration protects against SS atrophy in mice. Future studies are required to elucidate the etiology of RC muscle degeneration.

The impact of subpopulations of T lymphocytes on skeletal muscle is increasingly recognized. In agreement with the report that T lymphocytes are dispensable for the development of fatty degeneration in rats[66], RCT does not affect T lymphocyte count in mouse SS muscle (Figure 4d). However, RCT enriches the T lymphocyte pool in T_H (Fig 4h) and T_{reg} (Figure 4k) subpopulations. Multivariate analysis reveals injury type-specific differences in T lymphocyte populations not detected by individual measurements; TT+DN is discriminated from TT by increased overall T lymphocyte frequency and decreased T_{reg} frequency within the T_H pool (Fig 6). T_H are required for biomaterial-induced non-classical MΦ polarization and muscle regeneration after volumetric muscle loss[67]. T_{reg} aid repair of acutely injured skeletal muscle, possibly because they encourage MP to switch from classical to non-classical phenotypes [68]. Deficient T_{reg} accumulation in aged mice impairs

acute muscle regeneration [69]. Thus, enrichment of the T_H and T_{reg} fractions may confer protective effects on SS muscle after RCT. Moving forward, one intriguing hypothesis is that aging may exacerbate RCT-induced muscle degeneration due to deficient accumulation of T_H or T_{reg} subpopulations.

Conclusion

Profiling inflammatory cell infiltration of SS muscle is the first step toward elucidating the role of inflammation in RCT pathogenesis. Future studies should employ gain- and loss-of-function techniques to investigate the effects of distinct inflammatory subpopulations on SS degeneration.

Methods

Mouse model of massive rotator cuff injury

All animal procedures were conducted according to protocols approved by the Georgia Tech Institutional Animal Care and Use Committee. Male C57bl/6 mice aged 8 weeks were anesthetized using vaporized isoflurane (5% for induction and 2% for maintenance). Sustained-release buprenorphine was administered i.p. for analgesia. The right shoulder served as the injured group and the contralateral shoulder served as the uninjured control group. The right arm and chest were shaved, depilated, and sterilized using triplicate alternating washes of alcohol and chlorhexidine. Using a No. 11 scalpel blade, a lateral incision in the skin was made from the midline to the humeral head. Fascia was removed by blunt dissection. To expose the glenohumeral joint, the deltoid was split by making a ~5mm incision from the clavicle to the superior-lateral region of the humerus. The supraspinatus and infraspinatus tendons were sharply transected using the scalpel blade. The suprascapular nerve (SSN) was located by creating a small incision in the pectoralis major and bluntly dissecting the muscle. The SSN was severed using surgical microscissors. The deltoid and pectoralis were closed using absorbable 4-0 sutures and the skin was closed using wound clips. Mice were allowed unrestricted ambulation after awakening from anesthesia.

Flow cytometry

Mice were euthanized by CO₂ asphyxiation 7 days after injury to analyze cellular inflammation in the SS muscle via flow cytometry. To generate single cell suspensions, muscles were minced, digested in 1mg/mL collagenase IA for 45 min at 37°C, filtered through membranes with 40µm pore size, and resuspended in 3% FBS for immunostaining. Cell suspensions were immunostained for 30 min on ice followed by fixation in 2% PFA for 10 min and addition of CountBright™ Absolute Counting Beads. The following antibody panel was used: MerTK-PE (clone 108928; R&D Systems), CCR7-PE/Cy7 (clone 4B12; BioLegend), CD3-FITC (17A2; BioLegend), CD25-PerCP/Cy5.5 (clone PC61; BioLegend), Ly6C-APC (clone HK1.4, BioLegend), Ly6G-APC/Cy7 (clone 1A8; BioLegend), CD11c-BV421 (clone N418; BioLegend), CD11b-BV510 (clone M1/70; BioLegend), CD206-BV605 (clone C068C2; BioLegend), CD64-BV711 (clone X54-5/7.1; BioLegend), and CD4-BV785 (clone GK1.5; BioLegend). To discriminate positive versus negative expression of each marker, the fluorescence-minus-one (FMO) approach was used. Briefly, excess cell

suspension was immunostained with all antibodies except the marker of interest. The threshold of positive versus negative expression of the marker of interest was then set at the level of the most highly fluorescent events in the marker's corresponding channel. Samples were run on a BD FACS Aria IIIu cytometer and data was analyzed using FlowJo software. Cells were immunophenotyped according to the following gating scheme: macrophage, MerTK⁺CD64⁺; dendritic cell, NOT(MerTK⁺CD64⁺)CD11c⁺; monocyte, NOT(MerTK⁺CD64⁺)CD11c⁻CD11b⁺SSC^{lo}; neutrophil, Ly6G⁺SSC^{hi}; T lymphocyte, CD3⁺; helper T lymphocyte, CD3⁺CD4⁺; regulatory T lymphocyte, CD3⁺CD4⁺CD25⁺. Metrics reporting cell count normalized to muscle mass were calculated according to the equation $N = N_g \cdot B_a / (B_m \cdot M)$, where N_g is the number of cells counted in the gate-of-interest, B_a is number of counting beads added to the sample, B_m is the number of counting beads measured by the cytometer, and M is the wet muscle mass. Metrics reporting frequency were calculated by dividing the number of cells in the gate-of-interest by the number of cells in a particular upstream gate.

Principal component analysis

Unsupervised principal component analysis was performed using Matlab. The following 11 variables from flow cytometry were used as inputs to the model: muscle mass, MΦ (% of cells), MO (% of cells), DC (% of cells), T lymphocyte (% of cells), neutrophil (% of cells), CD206⁺ (% of MΦ), CCR7⁺ (% of MΦ), Ly6C^{lo} (% of MO), T_H (CD4⁺CD3⁺ % of CD3⁺), and T_{reg} (CD25⁺CD4⁺CD3⁺ % of CD4⁺CD3⁺). Ly6Chi (% of MO) was omitted because the value equals $1 - \text{Ly6C}^{\text{lo}}$. Data were scaled to unit variance and mean centered. Regarding quality of fit, Hoetelling's T2 and DModX are less than critical values for all observations. For PC1, $R^2 = 0.575$ and $Q^2 = 0.431$. For PC2, $R^2 = 0.166$ and $Q^2 = 0.064$.

Clodronate-liposome injection and myofiber diameter analysis

Intravenous administration of liposomal clodronate causes complete depletion of blood MO within 6 h followed by recovery of the Ly6C^{hi} and Ly6C^{lo} MO populations at 2 and 7 days, respectively [28]. To deplete circulating monocytes in the present study, liposomal clodronate (100μL per 10g of body weight; Dr. Nico van Rooijen, clodronateliposomes.org) or saline control was administered i.v. via jugular vein injection every 2–3 days according to the timeline in Figure 6A. Supraspinatus atrophy was assessed by wet mass and IHC analysis of myofiber cross-sectional area at day 21 post-injury. To prepare muscles for IHC, mouse vasculature was perfused with 0.9% saline followed by 4% paraformaldehyde and then muscles were harvested and snap frozen in liquid nitrogen-cooled 2-methylbutane. Muscle cross-sections (thickness: 10 μm) were acquired using a cryostat (Leica CryoStar NX70). Immunohistochemistry was performed on cross-sections by staining with goat anti-collagen IV (Millipore AB769) and donkey anti-goat conjugated to DyLight 594 (abcam ab96933). Fluorescence was visualized at 594 nm excitation using a Zeiss LSM 710 NLO confocal microscope. Regions of interest were selected to include most of the muscle cross-section interior and exclude the periphery. Semi-automated quantification of myofiber diameter was performed in ImageJ by manually thresholding pixel intensity, automatically detecting edges using the “Find Edges” process, and quantifying diameter of each myofiber using the “WandAutoMeasure” tool. To account for variation in number of myofibers

quantified, equal numbers of myofibers were randomly selected from each animal, pooled, and subjected to statistical analysis (n=964 across 4 animals per group).

Statistical analysis

In Figures 1–4, statistical comparisons were conducted in GraphPad Prism using repeated measures two-way ANOVA followed by Sidak multiple comparisons test. Figure 6 was analyzed by t-test (panel C) and Mann-Whitney test (panel E–G).

Abbreviations

MP	mononuclear phagocyte
MΦ	macrophage
MO	monocyte
DC	dendritic cell
T_h	helper T cell
T_{reg}	regulatory T cell
TT	tendon transection
TT+DN	tendon transection plus denervation
SS	supraspinatus
RCT	rotator cuff tear
SSN	suprascapular nerve

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Figure 1. Mononuclear phagocyte accumulation in supraspinatus muscle 7 days after rotator cuff injury

(A) Flow cytometry gating scheme to identify populations of mononuclear phagocytes after pre-gating for single cells. Mononuclear phagocyte count (B) is calculated as the sum of macrophages (C), monocytes (D), and dendritic cells (E) normalized to muscle mass. (F) Frequency of each mononuclear phagocyte population out of total cells. Closed circles represent the injured side; open circles, uninjured contralateral side. Statistical comparisons are conducted by repeated measures two-way ANOVA followed by Sidak multiple comparisons test (TT, n=5; TT+DN, n=4). *P<0.05, **P<0.01, ***P<0.001 comparing injury side to contralateral side. ^P<0.05, ^^P<0.01, ^^^P<0.001 comparing TT+DN to TT.

Figure 2. Macrophage subpopulations

(A) Flow cytometry gating scheme to identify CD206⁺ and CCR7⁺ macrophages. The fractions of CD206⁺ (B) and CCR7⁺ (C) cells within the macrophage pool are affected by rotator cuff injury. TT+DN injury increases the number of CD206⁺ and CCR7⁺ macrophages per gram of muscle (D). Closed circles represent the injured side; open circles, uninjured contralateral side. Statistical comparisons are conducted by repeated measures two-way ANOVA followed by Sidak multiple comparisons test (TT, n=5; TT+DN, n=4). *P<0.05, **P<0.01 comparing injured side to contralateral side. ^P<0.05, ^^P<0.01 comparing TT+DN to TT.

Figure 3. Monocyte subpopulations

(A) Flow cytometry gating scheme to identify Ly6C^{lo} and Ly6C^{hi} subpopulations of monocytes. The fraction of Ly6C^{lo} (B) and Ly6C^{hi} (C) cells within the monocyte pool are increased and decreased, respectively, by TT+DN injury. The numbers of Ly6C^{lo} and Ly6C^{hi} monocytes per gram of muscle are increased by injury (D). Closed circles represent the injured side; open circles, uninjured contralateral side. Statistical comparisons are conducted by repeated measures two-way ANOVA followed by Sidak multiple comparisons test (TT, n=5; TT+DN, n=4). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 comparing injury side to contralateral side. ^P<0.05, ^^P<0.01, ^^P<0.001 comparing TT+DN to TT.

Figure 4. Quantification of neutrophil and T lymphocyte populations

(A) Flow cytometry gating scheme to identify neutrophils (Ly6G⁺), T lymphocytes (CD3⁺), T_H(CD4⁺CD3⁺), and (CD25⁺CD4⁺CD3⁺). Cell count per gram of muscle of T_{reg} (I) is not significantly affected by neutrophils (B), T lymphocytes (D), T_H (F), and T_{reg} injury. TT increases neutrophil frequency out of total cells compared to TT+DN (C). TT decreases T lymphocyte frequency out of total cells compared to uninjured contralateral (J) frequency out of total cells. The fractions of (E). Neither injury affects T_H (G) or T_{reg} (K) within the T lymphocyte pool are increased by injury. The fraction of T_H (H) and T_{reg} pool is unaffected by injury (L). Statistical comparisons are conducted T_{reg} within the T_H by repeated measures two-way ANOVA followed by Sidak multiple comparisons test (TT, n=5; TT+DN, n=4).

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 comparing injured side to contralateral side. ^P<0.05, ^^P<0.01, ^^P<0.001 comparing TT+DN to TT.

Figure 5. Unsupervised principal component analysis of flow cytometry quantification

(A) Loadings (weight coefficients) plot of the 11 input variables in the reduced principal component space. Input variables are: muscle mass, MΦ (% of cells), MO (% of cells), DC (% of cells), T lymphocyte (% of cells), neutrophil (% of cells), CD206⁺ (% of MΦ), CCR7⁺ (% of MΦ), Ly6C^{lo} (% of MO), T_H (CD4⁺CD3⁺ % of CD3⁺), and T_{reg} (CD25⁺CD4⁺CD3⁺ % of CD4⁺CD3⁺). (B) Injury conditions are separated by the model in the scores plot (TT, n=5; TT+DN, n=4). Quality-of-fit metrics are provided in Methods.

Figure 6. Liposomal clodronate accelerates muscle atrophy

Liposomal clodronate is delivered intravenously at 2–3 day intervals following TT+DN injury (A). To assess muscle atrophy, supraspinatus mass (B) and percent change in mass between injured and uninjured contralateral supraspinatus (C) are measured at day 21 (Saline, n=4; Clod-lip, n=5). Myofiber cross-sectional area is visualized by anti-collagen IV IHC (D) and quantified using ImageJ (E–G) (n=964 across 4 animals per group). Statistical comparisons are conducted by two-way ANOVA with Sidak post-hoc test (B), t-test (C), and Mann-Whitney rank test (E–G). ^P<0.05, *P<0.05, ***P<0.001, ****P<0.0001. Scale bar: 100 μm .