

AWARD NUMBER: W81XWH-17-1-0655

TITLE: Targeting Neutrophil Extracellular Traps to Mitigate Post-Traumatic Lower-Extremity Soft Tissue Injury

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REPORT DATE: JANUARY 2021

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE JANUARY 2021		2. REPORT TYPE Final Report		3. DATES COVERED 30 Sep 2017– 29 Sep 2020	
4. TITLE AND SUBTITLE Targeting Neutrophil Extracellular Traps to Mitigate Post-Traumatic Lower-Extremity Soft Tissue Injury				5a. CONTRACT NUMBER W81XWH-17-1-0655	
				5b. GRANT NUMBER OR160105	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Benjamin Levi, MD E-Mail: Benjamin.Levi@UTSouthwestern.edu				5d. PROJECT NUMBER 0011039268	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Regents of the University of Michigan Kathryn Dewitt 503 Thompson St Ann Arbor MI 48109-1340				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Tissue damage from musculoskeletal trauma often includes a secondary ischemia-reperfusion (IR) injury, which results in fibrosis and loss of function in the skeletal muscle. Here, we investigate strategies to mitigate skeletal muscle fibrosis caused by IR injury by targeting neutrophil extracellular trap (NETs) formation via PAD4 and TLR9 mechanisms. We show that muscle fibrosis is largely independent of PAD4 and TLR9 mediated NET formation. Importantly, we identify administration of FDA-approved Plaquenil(hydroxychloroquine) as a promising treatment strategy to reduce fibrosis. In mouse models, treatment with Plaquenil decreased skeletal muscle fibrosis and improved myofiber regeneration following IR injury.					
15. SUBJECT TERMS Ischemia reperfusion, neutrophils, neutrophil extracellular trap, skeletal muscle fibrosis, PAD4, TLR9, hydroxychloroquine, Cl-amidine					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	55	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	3
2. Keywords	3
3. Accomplishments	3
4. Impact	19
5. Changes/Problems	20
6. Products	20
7. Participants & Other Collaborating Organizations	20
8. Special Reporting Requirements (Quad Chart)	22
9. Appendix A: Novel FDA Approved Therapy to Mitigate Ischemia-Reperfusion Induced Fibrosis <i>Abstract accepted to Military Health Science Research Symposium 2020</i>	
10. Appendix B: “Edwards NJ, Hwang C, Marini S, et al. The role of neutrophil extracellular traps and TLR signaling in skeletal muscle ischemia reperfusion injury. <i>The FASEB Journal</i> . 2020. <i>In Press</i> ”	

1. Introduction

Musculoskeletal extremity injuries are complicated by heightened risk for acute vascular injury with subsequent ischemia and tissue damage. Reperfusion after ischemia further exacerbates tissue damage and worsens microvascular injury and muscle function. Traumatic injury and ischemia-reperfusion injury also leads to the formation of neutrophil extracellular traps (NETs) by neutrophils recruited to the injured tissue during the initial immune response. While important for host defense, excessive NET production (NETosis) can ultimately be deleterious to tissue regeneration. Our central hypothesis is that NETosis mediates the damaging effects of IR injury on endothelial cell dysfunction and musculoskeletal soft tissues. This proposal will validate a therapeutic approach targeting either the initial/primary formation of NETs via PAD4 inhibition or the propagation of NETs (secondary NETosis) via inhibition of TLR9 signaling. We will demonstrate that NETs mediate post-traumatic IR extremity inflammation, cause endothelial cell dysfunction and skeletal muscle injury during reperfusion and that inhibition of PAD4 (GSK199, CI-Amidine) or TLR9 (ODN-2088, Plaquenil) aimed at modulating NETosis is protective against IR related musculoskeletal soft tissue injury.

2. Keywords

Ischemia-reperfusion injury, skeletal muscle fibrosis, neutrophil extracellular traps, CI-amidine, Plaquenil, hydroxychloroquine, trauma, PAD4, TLR9, neutrophils, fibro-adipogenic progenitor cells

3. Accomplishments

What were the major goals of the project?

Regulatory Tasks:

Subtask 1: IACUC/ACURO Approval

Status: Status: IACUC protocol number: PRO00005909/PRO00007930 approved 2017-09-20.

ACURO protocol number: OR160105 initial approval 2017-10-11, rewrite 2018-03-27, minor amendment approved 2018-07-12.

Specific Aim 1: To demonstrate that NETs exacerbate skeletal muscle IR injury and to validate that PAD4 inhibition with GSK199 or CI-Amidine prevents NETosis, endothelial cell damage and soft tissue injury after hind limb ischemic trauma without impaired wound healing. (months 1-20)

Status: Completed

Subtask 1: Ischemia reperfusion mouse model on C57BL/6 mice to assess the effect of systemic administration of PAD4 inhibition on hind limb perfusion, muscle fibrosis, platelet adhesion/aggregation (microthrombosis), neutrophil recruitment/NETs formation in vivo using laser Doppler imaging, ultrasound imaging and intravital fluorescence microscopy. n=28 mice. (months 4-12)

Status: Completed

Subtask 2: Analysis of inflammation, endothelial dysfunction, microthrombi formation, and muscle fibrosis to be done using histology, flow cytometry, Western blot analysis, and serum analysis. n=290 mice. (months 4-18)

Status: Completed

Subtask 3: Assess muscle force contractile properties after IR injury with and without PAD4 inhibitor treatments. n=18 mice. (months 4-18)

Status: Not Indicated

Milestone: Completion of PAD4 IR studies and data analysis. Preparation and submission of peer-reviewed manuscripts. (month 20)

Status: Completed

Specific Aim 2: To decrease endothelial cell dysfunction and to prevent progression of ischemia reperfusion soft tissue injury using FDA-approved TLR9 inhibitor (Plaquenil) and ODN-2088 to mitigate secondary NET propagation (NET induced NETosis). (months 18-24)

Status: Completed

Subtask 1: In vivo assessment of TLR9 inhibition on hind limb perfusion, muscle fibrosis, platelet adhesion/aggregation (microthrombosis), neutrophil recruitment/NETs formation in vivo using laser Doppler imaging, ultrasound imaging and intravital fluorescence microscopy. n=28 mice (months 18-24)

Status: Completed

Subtask 2: Analysis of inflammation, endothelial dysfunction, microthrombi formation, and muscle fibrosis to be done using histology, flow cytometry, Western blot analysis, and serum analysis. n=290 mice. (months 18-24)

Status: Completed

Subtask 3: Assess muscle force contractile properties after IR injury with and without TLR9 inhibitor treatments. n=18 mice (months 18-24)

Status: Not indicated

Milestone: Completion of IR induced muscle extremity injury studies and data analysis.

Preparation and submission of peer-reviewed manuscripts. (months 22-24)

Status: Completed

What was accomplished under these goals?

Specific Aim 1: To demonstrate that NETs exacerbate skeletal muscle IR injury and to validate that PAD4 inhibition with GSK199 or Cl-Amidine prevents NETosis, endothelial cell damage and soft tissue injury after hind limb ischemic trauma without impaired wound healing.

1. Ischemia Reperfusion Injury

1.1 Ischemia Reperfusion Injury Leads to Skeletal Muscle Fibrosis

Tissue damage from ischemia reperfusion (IR) injury occurs when blood supply is restored to the tissue following a period of ischemia, such as after a traumatic injury and subsequent repair. Tissue damage results not only from the period of ischemia, but also from the subsequent restoration of blood flow. Causes of IR injury have not been completely elucidated, however the main factors are believed to be the creation of reactive oxygen species in the injury site and a vigorous inflammatory response in the affected tissue, especially the infiltration of activated neutrophils (1, 2). Several studies have investigated varying aspects of IR injury in skeletal muscle, however a complete, detailed characterization of damage caused by IR injury will be valuable to more fully understand the mechanisms of IR injury and to study potential therapeutic options following IR injury (2-4).

In order to fully characterize the extent of skeletal muscle injury following IR injury, we have analyzed skeletal muscle injury and regeneration in a mouse model of hind limb skeletal muscle IR injury. Hind limb IR injury was induced in male C57BL/6 mice by occluding the femoral artery using a microvascular clamp for 3 hours followed by reperfusion until time of sacrifice. The tibialis anterior muscle was collected, flash frozen, sectioned, and stained in order to analyze regeneration. **Figure 1** shows the timeline of skeletal muscle regeneration after IR injury at 3, 7, and 14 days post injury (DPI). As shown by hematoxylin & eosin (H&E) stain, during the first three days after injury the majority of myofibers are necrotic and a significant immune cell infiltration is present. At five days, myofiber regeneration (characterized as possessing a centralized nuclei) begins near the edge of the muscles, with necrotic myofibers are still present in the center of the muscle. By 7 DPI, myofibers are largely in the regenerative state. By 14 DPI, muscles appear morphologically similar to uninjured muscles, however centralized nuclei in the myofibers are still present. As shown by picrosirius red staining, which stains collagens, initial collagen depositions are present in an unorganized manner by 3 DPI. By 7 DPI, fibrotic collagen depositions are present around the forming myofibers. By 14 DPI, collagen deposition is reduced from day 7 and the collagen staining pattern is more similar to uninjured muscles.

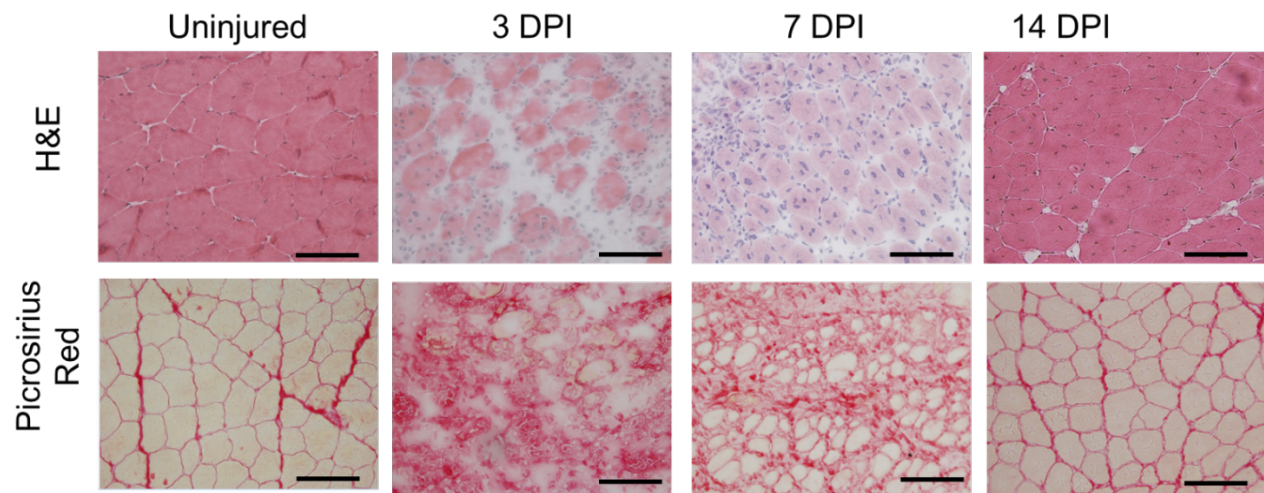


Figure 1: Histological characterization of IR injury in the C57BL/6 mouse. Representative H&E and picrosirius red stains of histological sections of TA following 3 hr IR injury in the C57BL/6 mouse at 3, 7, and 14 DPI. $n \geq 3$ at all time points. All scale bars indicate 100 μ m.

1.2 Neutrophil Extracellular Traps Form in Skeletal Muscle after Ischemia Reperfusion Injury

Since their discovery by Brinkmann and colleagues in 2004, neutrophil extracellular traps (NETs) have been implicated in a wide variety of diseases (5). Recent studies have also implicated in NETs in IR injury, indicating that following ischemic injury, stimulated neutrophils can lead to the production of NETs at the site of injury (2, 3). Initial reports have detected NETs in skeletal muscle following IR injury, although less is known about the specific roles and mechanisms through which NETs affect skeletal muscle fibrosis (3). In order to study the role of NETs in skeletal muscle injury, an IR injury was induced in a C57BL/6 mouse and muscle sections of the TA were collected, histologically processed, and stained with immunofluorescence (IF) techniques for citrullinated histone 3 (H3Cit), myeloperoxidase (MPO), and nuclei (Hoechst). Neutrophil extracellular traps were identified as events in which MPO was co-localized with the nucleus, co-localization of H3Cit with MPO, and/or a web-like structure of nuclei, MPO, and H3Cit, representing extruded genetic matter into the extracellular space. Three high powered representative images (63x) were taken on one muscle section per animal ($n=6$ per time point) in similar areas of the muscle for each section using a Leica SPX8 confocal microscope. The number of NETs per region of interest (ROI) were quantified by trained personnel and an average was obtained per animal. As shown in **Figure 2**, robust NET formation was detected in IR injured skeletal muscle at 1 and 3 DPI. NETs were not present in uninjured muscle.

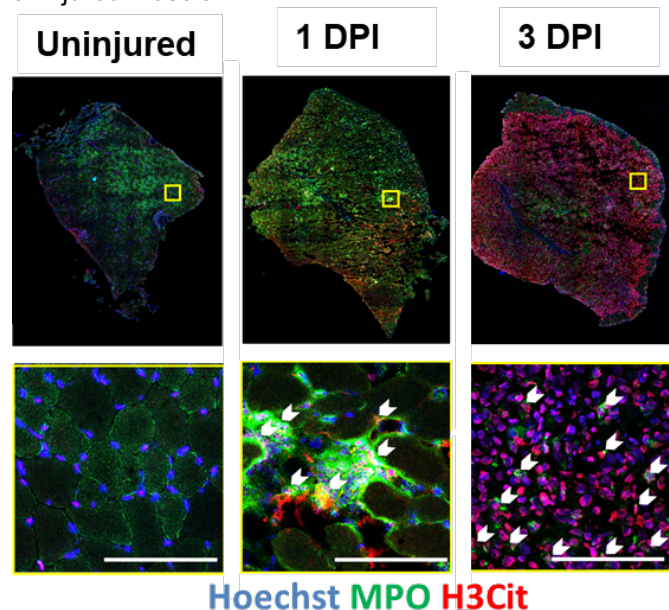


Figure 2: Neutrophil extracellular traps form after IR injury in mouse skeletal muscle. Immunofluorescent stains for NETs at 1 and 3 DPI in the C57BL/6 strain. White arrowheads indicate examples of NETs. NETs were not present in uninjured muscle. Scale bars indicate 100 μ m. Representative of $n \geq 6$ per group.

Given the NET formation in the skeletal muscle following IR injury, and knowing NETs exacerbate injury states on other models and tissues, we hypothesized that NETs play a role in the development of fibrosis. In order to test this theory, injured mice were treated with two different classes of drugs to decrease NET formation. First, mice were treated with peptidyl arginine deiminase inhibitors (PAD4) to decrease initial NETs induced via suicidal NETosis. Second, mice were treated with modulators of TLR7/8/9 signaling in order to decrease secondary NET-induced NETosis. Muscle regeneration, fibrosis, and NET formation were analyzed in the tibialis anterior following IR injury and treatment.

1.3 Administration of PAD4 inhibitors to mitigated muscle fibrosis following IR injury

Several studies have shown that the PAD4 is required for NET formation in suicidal NETosis via citrullination of histone 3 during NET formation (6-9). Accordingly, it is also known that mice lacking PAD4 do not form NETs via suicidal NETosis (6). Cl-amidine, a small molecule inhibitor of PAD4, has been shown to decrease NET formation in a variety of disease models including lupus (10), collagen-induced arthritis (11), multiple sclerosis (12), and others. Given that NETs formed in the skeletal muscle of this mouse model of IR injury demonstrate citrullination of histone H3 (**Fig 2**), we first sought to investigate the role of NETs formed via the PAD4 mechanism.

1.3.1 Administration of Cl-amidine does not mitigate muscle fibrosis following IR injury and muscle fibrosis following IR injury is PAD4 independent

Full details of findings following the administration of Cl-amidine to mitigate muscle fibrosis following IR injury are reported in Edwards, et al (2020) (13). Briefly, we found that while administration of 10 mg/kg Cl-amidine significantly reduced NET formation at 3 DPI (from 24.1 ± 11.7 to 3.9 ± 2.7 following treatment [NETs per 63x ROI, n=3 per group; $P < 0.05$; **Fig 3A**]), treatment did not improve muscle regeneration and muscle fibrosis was not decreased. Mice treated with Cl-amidine presented with an increased frequency of myofibers with small CSA and Feret diameter ($P < 0.0001$, both analyses), indicating that administration of Cl-amidine hindered muscle regeneration at 7 DPI compared to vehicle (**Fig. 3B**). Similarly, Cl-amidine did not have an effect on muscle fibrosis at 7 DPI compared to the vehicle ($47.7\% \pm 11.1$ vs $50.6\% \pm 5.5$ vs $17.5 \pm 0.41\%$; vehicle, Cl-amidine, uninjured TA respectively; mean $\pm$ SD $P = 0.8$, n=3-6/group; ordinary one-way ANOVA plus Tukey's test), (**Fig. 3C**). As pharmacological intervention may not completely inhibit the PAD4 signaling, we next sought to investigate this phenomenon in a transgenic mouse lacking PAD4. To ensure a robust injury and closely mimic the polytrauma injuries that often accompany IR in battlefield injuries, IR injury was performed as described and 10 μ L of 3 mg/ml ctx was also injected into the injured tibialis anterior immediately following clamp release in the transgenic mouse studies. Findings in the PAD4^{-/-} mice were similar to those treated with Cl-amidine. PAD4^{-/-} mice did not show increased regeneration or decreased muscle fibrosis following IR injury, as shown in **Fig. 3D-E**. To identify if any treatments or genotypes provided protective effects on the myofibers, we compared the percent of myofibers possessing centralized nuclei (indicating regenerating myofibers) between groups and the percent of myofibers exhibiting nuclei on the periphery of the myofiber (indicating an uninjured/protected myofiber). We found similar myofiber composition between vehicle and Cl-amidine treatment, as shown in **Fig 3F**. Similarly, both C57BL/6 and PAD4^{-/-} muscles were comprised of nearly 100% regenerating fibers without substantial peripherally-located nuclei (**Fig 3G**). Taken together, these experiments indicate that while PAD4 inhibition does decrease NET formation in skeletal muscle following IR injury, mice treated with Cl-amidine have similar injury to mice treated with vehicle with hindered myofiber regeneration and similar fibrotic deposition in the skeletal muscle. Since decreasing NETs via PAD4 inhibition and genetic deletion did not mitigate fibrosis, we conclude that the formation of skeletal muscle fibrosis after IR injury is largely independent of PAD4 mediated NET formation.

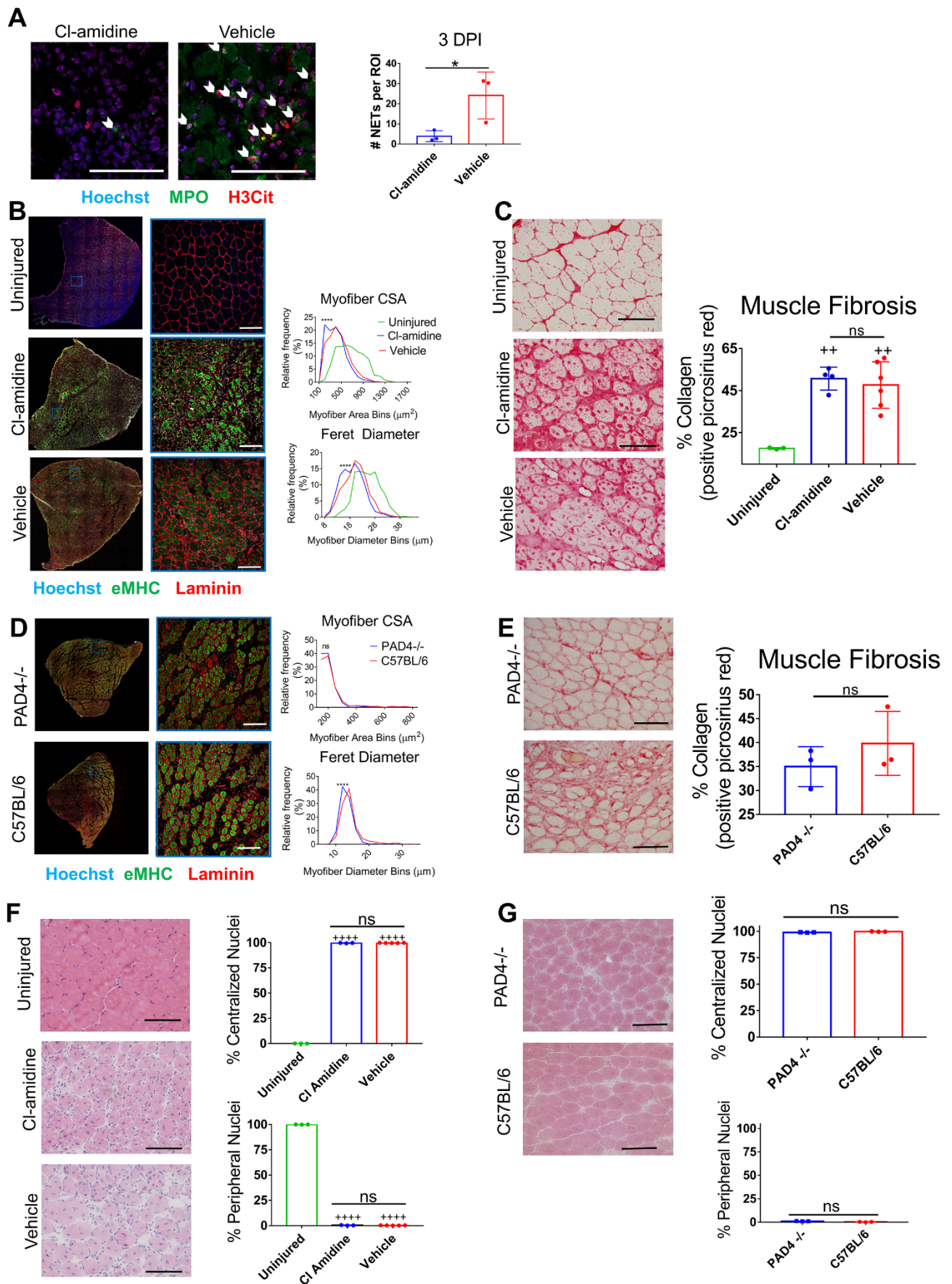


Figure 3: Muscle fibrosis is independent of PAD4 mediated NETosis. A) Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with Cl-amidine or vehicle. Treatment with Cl-amidine significantly decreased NET formation. B) Representative images

of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle stained with embryonic myosin heavy chain (eMHC, to indicate regenerating myofibers) and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber cross sectional area (CSA) and Feret diameter at 7 DPI, indicating muscles with Cl-amidine treatment have significantly increased frequency of smaller myofibers as indicated by CSA or diameter on x-axis. C) Representative histological sections of picrosirius red staining at 7 DPI of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle. ImageJ quantification of positive picrosirius red staining in the same groups. D) Representative sections of PAD4^{-/-} mice or C57BL/6 wild type control injured with IR+ctx stained with eMHC and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter at 7 DPI, indicating PAD4^{-/-} muscles have significantly increased frequency of smaller diameter myofibers. E) Representative histological sections of picrosirius red staining at 7 DPI of PAD4^{-/-} or C57BL/6 wild type control injured with IR+ctx. ImageJ quantification of positive picrosirius red staining in the same groups. F) Representative images of H&E stained TA 7 DPI of uninjured and treated with Cl-amidine or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. G) Representative images of H&E stained muscle 7 DPI of PAD4^{-/-} and C57BL/6 mice. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. + indicates difference compared to uninjured group. All scale bars indicate 100 μ m. * indicates $P<0.05$; ++ indicates $P<0.005$; **** and +++++ indicate $P<0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means $\pm$ standard deviation. Each point represents one animal. Figure from (13).

1.3.2 Administration of GSK-199 does not mitigate muscle fibrosis following IR injury

GSK-199 is also a potent PAD4 inhibitor (8). In order to investigate the effects of GSK-199 treatment after IR injury, 30 mg/kg GSK-199 in phosphate buffered saline (PBS), or vehicle control (PBS) was administered via subcutaneous injections daily. Mice were sacrificed and myofiber regeneration and fibrosis was quantified at 7 DPI. Mice treated with GSK-199 had a similar percentage of regenerating myofibers at 7 DPI. This indicates that the initial injury was similar in both groups and treatment with GSK-199 does not provide protection from injury. Similarly, GSK-199 did not decrease muscle fibrosis at 7 DPI compared to vehicle control ($47.7\% \pm 11.1$ vs $49.5\% \pm 4.4$ vs $17.5 \pm 0.41\%$; vehicle, GSK-199, uninjured TA respectively; mean $\pm$ SD $P=0.9$, $n=3-6$ /group; ordinary one-way ANOVA plus Tukey's test). **Fig. 4** shows representative examples of muscle regeneration and fibrosis in animals treated with GSK-199 or vehicle control.

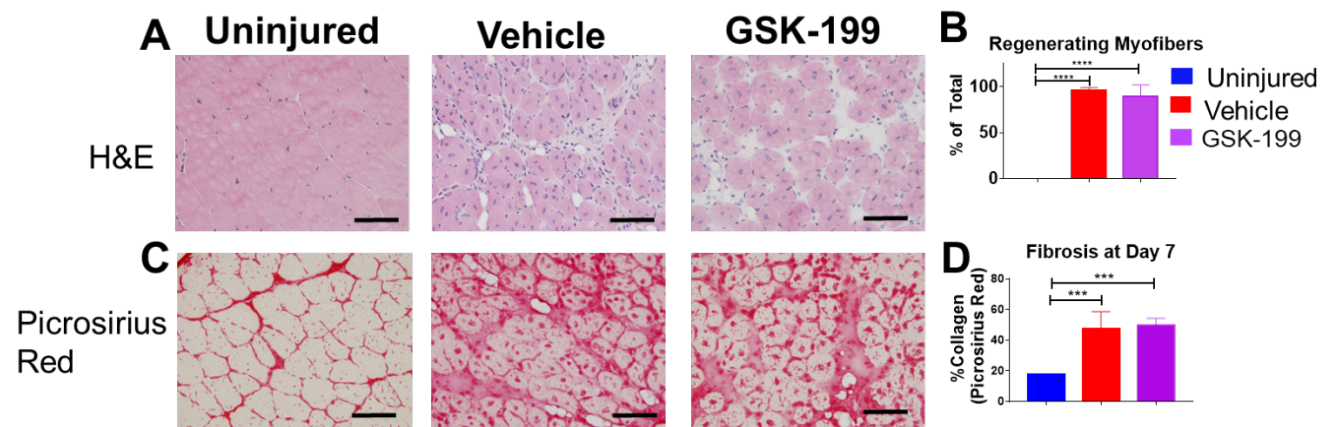


Figure 4: Treatment with GSK-199 does not increase regeneration or decrease fibrosis following muscle IR injury. A) H&E staining of uninjured or treated TA at 7 DPI and B) quantification of regenerating (centralized nuclei) myofibers as a percentage of total myofibers. C) Picrosirius red staining of uninjured or treated TA at 7 DPI and D) quantification of fibrosis based on picrosirius red quantification. All images and quantifications representative of $n \geq 3$ per group. Scale bar is 100 μ m in all images. Significance analyzed with ordinary one-way ANOVA to compare all groups to every other group, followed by Tukey test to correct for multiple comparisons; *** indicates $p<0.0002$ **** indicates $p<0.0001$.

Key Findings or Accomplishments for Specific Aim 1

First, we established that our model of hind limb IR injury via occlusion of the femoral artery and vein for three hours results in robust NET formation at 1 and 3 DPI, with no NETs present in an uninjured muscle. Following NET formation, muscle fibrosis is present at 7 DPI, and decreases by 14 DPI. Staining for NETs showed that NET formation occurs via citrullination of H3, a mechanism dependent on PAD4. Therefore, we hypothesized that the formation of NETs may contribute to the skeletal muscle fibrosis developed after IR injury and sought to

decrease NET formation via PAD4 inhibition by administering small molecule Cl-amidine or GSK-199. As expected, Cl-amidine treatment did significantly decreased NET formation, however Cl-amidine inhibited myofiber regeneration and did not affect skeletal muscle fibrosis at 7 DPI. Similarly, treatment with another PAD4 inhibitor, GSK-199, did not mitigate skeletal muscle fibrosis either. Because small molecule inhibitors do not completely inhibit all PAD4 signaling, we next induced a transgenic mouse lacking PAD4 to further characterized the role of PAD4 induced NETosis in skeletal muscle after IR injury. We found that PAD4^{-/-} mice do not present with decreased fibrosis or increased regeneration after IR traumatic injury. Together, we concluded that skeletal muscle fibrosis following IR injury is independent of NETosis via the PAD4 pathway.

Specific Aim 2: To decrease endothelial cell dysfunction and to prevent progression of ischemia reperfusion soft tissue injury using FDA-approved TLR9 inhibitor (Plaquenil) and ODN-2088 to mitigate secondary NET propagation (NET induced NETosis).

2. Administration of TLR9 inhibitors to mitigate muscle fibrosis following IR injury

Recent reports by our lab and others have shown that toll-like receptor (TLR) signaling is also implicated in NETosis outside of suicidal NETosis mediated by PAD4 (14-16). TLR7/8/9 have been linked to secondary self-NET propagation through stimulation by NETs released into the tissue during the initial immune response or free nucleic material present in the tissue due to the initial traumatic insult (16, 17). Having shown a robust NET formation in the injured skeletal muscle of wild type mice, we were also interested in further investigating the role of TLR signaling in NET formation in IR injury. We hypothesized that inhibition of TLR9 with ODN-2088 and hydroxychloroquine (Plaquenil) may decrease NET formation via a TLR signaling mechanisms following IR injury to decrease NET formation and muscle fibrosis.

2.1 Administration of ODN-2088 to mitigate muscle fibrosis following IR injury

ODN-2088 is an inhibitory CpG oligodeoxynucleotide (ODN) with specificity towards murine TLR9. In these experiments, we hypothesize that inhibition of TLR9 may decrease muscle fibrosis after IR injury and act to mitigate secondary NET propagation in the NET induced NETosis model. In order to investigate this hypothesis, mice underwent IR injury and were treated with 150 ng/g ODN-2088 or ODN-2088 control starting three days prior to injury and daily for seven days (study end). Muscle regeneration and fibrosis was investigated in the tibialis anterior muscle as previously described. A significant difference in muscle regeneration was not seen between ODN-2088 and control treated mice based on myofibers that exhibit centralized nuclei, which indicates that the initial injury was similar in the two groups and TLR9 inhibition did not protect myofibers from injury. Furthermore, a significant difference in muscle fibrosis was not present between treated and untreated mice at 7 DPI ($37.2\% \pm 7.3$ vs $42.3\% \pm 3.9$ vs $25.6 \pm 8.0\%$; ODN control, ODN-2088, uninjured TA respectively; mean $\pm$ SD $P=0.4$, $n=4-6$ /group; ordinary one-way ANOVA plus Tukey's test). **Fig. 5** shows representative examples of muscle regeneration and fibrotic disposition in animals treated with ODN-2088 and the respective quantifications.

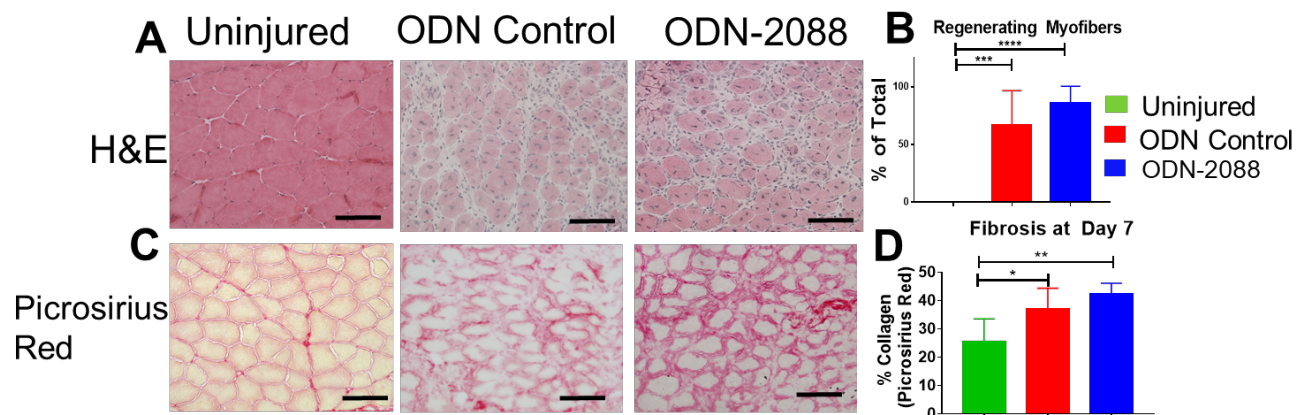


Figure 5: Treatment with ODN-2088 mitigate fibrosis following muscle IR injury. A) H&E staining of uninjured or treated TA at 7 DPI and B) quantification of regenerating (centralized nuclei) myofibers as a percentage of total myofibers. C) Picrosirius red staining of uninjured or treated TA at 7 DPI and D) quantification of fibrosis based on picrosirius red quantification. All images and quantifications representative of $n \geq 3$ per group. Scale bar is 100 μ m in all images. A significant difference fibrosis between ODN-2088 control or ODN-2088 treatment was not present at 7 DPI. Significance analyzed with ordinary one-way ANOVA to compare all groups to every other group, followed by Tukey test to correct for multiple comparisons; *** indicates $p < 0.0002$ **** indicates $p < 0.0001$

2.2 Hydroxychloroquine (Plaquenil) mitigates muscle fibrosis and increases muscle regeneration following IR injury

Next, another TLR7/8/9 inhibitor, hydroxychloroquine, was investigated. Hydroxychloroquine (HCQ), originally an anti-malarial drug, has recently been shown to have potential beneficial effects for IR injury in several organs (18-20). HCQ is also a potent inhibitor of the TLR7, 8, 9 pathways, which may play a role in NETosis as well (14). Here, we hypothesize that administration of HCQ in skeletal muscle IR injury may improve muscle recovery following IR injury in the skeletal muscle. IR injury was induced as previously described. Mice were treated with hydroxychloroquine sulfate at 60 mg/kg daily via intraperitoneal injection or with vehicle control (phosphate buffered saline, PBS) starting on the day of injury.

Full details of findings following the administration of HCQ to mitigate muscle fibrosis following IR injury are reported in Edwards, et al (2020) (13). Briefly, we found that while HCQ does not significantly decrease NET formation (NETs per 63x ROI: HCQ: 4.5 ± 1.6 , vehicle: 7.4 ± 3.2 ; $n = 6/\text{group}$; $P = .08$, unpaired t test), (**Fig. 6A**), treatment with HCQ significantly reduced muscle fibrosis at 7 DPI ($40.5\% \pm 4.0$ vs $30.4\% \pm 5.4$ vs $18.5\% \pm 2.3$; vehicle, HCQ, uninjured, respectively; $n=6/\text{group}$, $P<0.01$, ordinary one-way ANOVA plus Tukey's test; **Fig. 6B**) and increases myofiber regeneration based on myofiber cross-sectional area (**Fig. 6C**). In order to determine if the differences seen in fibrosis and regeneration in the HCQ treated animals was a result of less severe initial injury, we again compared the percentage of regenerating (centralized nuclei) and uninjured/protected (edge nuclei) myofibers in each treatment group to determine if HCQ provided protective effects on the myofibers. The percentage of regenerating myofibers was not significantly different between vehicle and HCQ treated animals ($99.32\% \pm 0.67$ vs $96.6\% \pm 3.7$ vs 0% ; vehicle, HCQ, uninjured, respectively, $n=6/\text{group}$, ANOVA followed by Tukey's test), as shown in **Fig 3D**. HCQ did not prevent initial injury to the myofibers either, as the composition of peripherally-located myofibers was not significantly different between vehicle and HCQ treated animals ($0.67\% \pm 0.67$ vs $3.41\% \pm 3.68$ vs 100% ; vehicle, HCQ, uninjured, respectively, $n=6/\text{group}$, ANOVA followed by Tukey's test) (**Fig. 3D**). Through these studies, **we have identified that treatment with FDA-approved HCQ mitigated muscle fibrosis and improved myofiber regeneration following IR injury, suggesting HCQ may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury.**

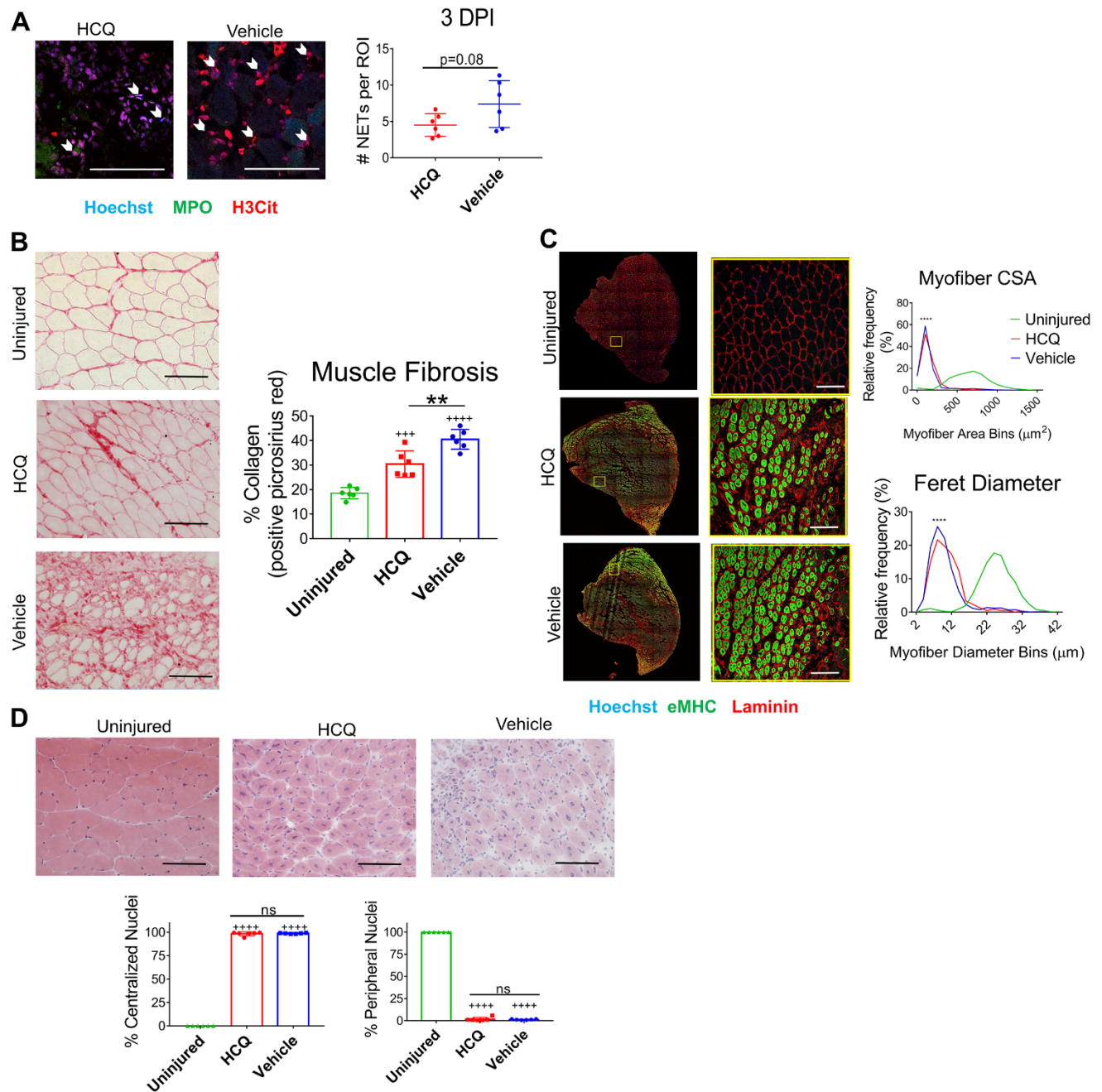


Figure 6: HCQ decreases muscle fibrosis and increases myofiber regeneration following IR injury. A) Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with HCQ or vehicle. Quantification shows HCQ treatment modestly decreased NET formation. B) Representative histological sections of picosirius red staining of TA at 7 DPI of C57BL/6 mice uninjured and treated with HCQ or vehicle. ImageJ quantification of positive picosirius red staining in the same groups shows HCQ treatment decreased collagen deposition. C) Representative images of C57BL/6 mice uninjured TA and treated with HCQ or vehicle stained with eMHC and laminin at 5 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter, indicating muscles with vehicle treatment have significantly increased frequency of small CSA and feret diameter. D) Representative images of H&E stained TA 7 DPI in uninjured and treated with HCQ or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. All scale bars indicate 100 μm . + indicates difference compared to uninjured group. ** indicates $P<0.01$; ++ indicates $P<0.005$; *** and ++++ indicate $P<0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means $\pm$ standard deviation. Each point represents one animal. Figure from Edwards, et al. (2020) (13).

2.3 Muscle fibrosis following IR injury is TLR9 independent

Having shown that HCQ, a TLR7/8/9 inhibitor decreased fibrosis, we sought to more thoroughly investigate the role of TLR9 signaling in IR injury. In order to do this, we utilized a transgenic mouse model of systemic TLR9 deletion (TLR9^{-/-}). We first investigated the ability of TLR9^{-/-} mice to form NETs after IR injury. TLR9^{-/-} mice and control BALB/cJ wild type mice underwent IR injury as previously described, except with a one hour ischemic period due to strain differences previously reported in progress reports. Mice were sacrificed at 3 DPI and NETs were investigated in the tibialis anterior by immunofluorescent staining for MPO, H3Cit, and Hoechst and NETs were identified and quantified as previously described. TLR9^{-/-} did not show a significant decrease in NET formation at 3 DPI (NETs per 63x region of interest, average $\pm$ SD, BALB/cJ: 40.2 ± 15.3 ; TLR9^{-/-}: 28.6 ± 9.9 ; $p=0.15$; $n=6$ per group, unpaired t-test), shown in **Fig. 7**. As NET formation is known to be mediated by additional pathways outside of TLR signaling, namely PAD4, modest decreases were expected. The modest decrease in NETs seen in the TLR9^{-/-} mice as well as in mice treated with TLR7/8/9 inhibitor Plaquenil further indicates that NET formation is likely partially, but not wholly, mediated by TLR signaling following traumatic injury.

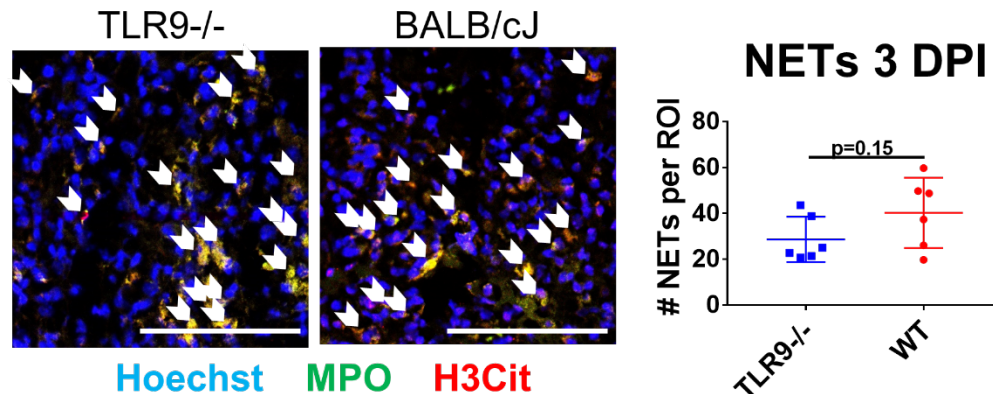


Figure 7: Formation of NETs after IR injury is independent of TLR9. TLR9 deletion does not significantly decrease NET formation in the skeletal muscle at 3 days post injury (DPI) after ischemia reperfusion injury. Representative images of TLR9^{-/-} and BALB/cJ wild type control tibialis anterior sections stained for NETs, left. Scale bars indicate 100 μ m. Right, quantification of average number of NETs per ROI. Analyzed with unpaired t-test. $n=6$ mice per group. Each point represents one animal (average of 3 ROIs per animal).

2.4 Muscle fibrosis following IR injury is independent of TLR9

After showing that administration HCQ, a TLR7/8/9 inhibitor, decreased muscle fibrosis following IR injury, we further investigated the role of TLR9 specifically in muscle fibrosis. Again, TLR9^{-/-} and BALB/cJ wild type control mice underwent IR injury with one-hour ischemic period and reperfusion for 7 days. Mice were sacrificed, tibialis anterior muscles collected, processed, and stained with picrosirius red to identify collagen deposition. ImageJ was used to quantify the amount of fibrosis based on the area of muscle positive for picrosirius red. At 7 DPI, a significant difference was not present in the fibrotic deposition between TLR9^{-/-} mice and the wild type control, as shown in **Fig. 8A** (BALB/cJ wild type: $43.7\% \pm 5.3$; TLR9^{-/-}: $40.2\% \pm 4.50$, $p=0.35$, $n=4$ per group, unpaired t-test). TLR9^{-/-} mice also had similar percentage of regenerating myofibers as the BALB/cJ mice, indicating that TLR9 deletion did not provide protective effects on the myofibers, as shown in **Fig. 8B**.

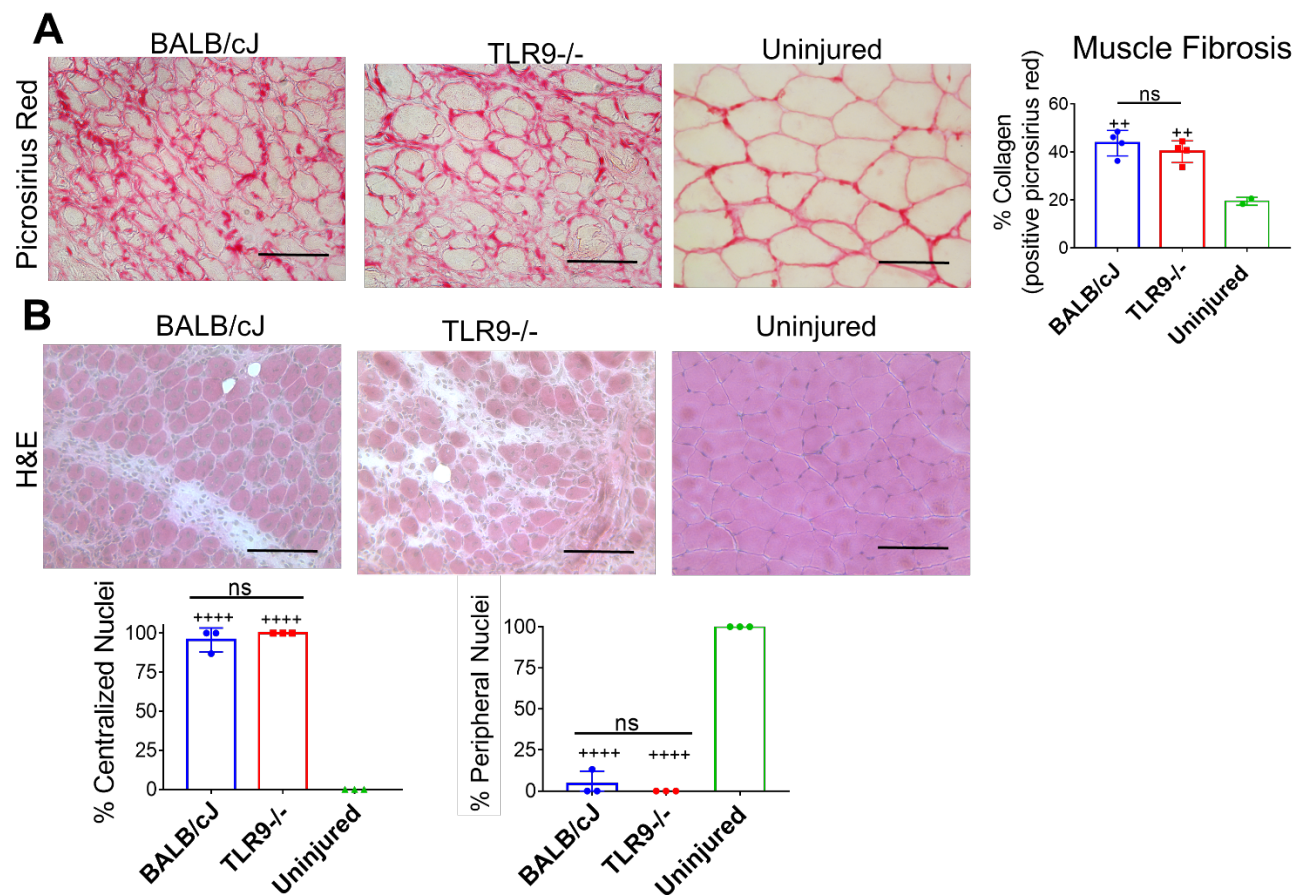


Figure 8: Muscle fibrosis is independent of TLR9. Muscles from TLR9^{-/-} mice have similar fibrosis to those of BALB/cJ wild type control mice at 7 DPI. A) Representative histological sections of picrosirius red staining at 7 DPI of BALB/cJ wild type, TLR9^{-/-}, and uninjured TA. ImageJ quantification of positive picrosirius red staining in the same groups. B) Representative images of H&E stained muscle 7 DPI of BALB/cJ, TLR9^{-/-}, or uninjured mice. Quantification of percentage of total myofibers with centralized (regenerating myofiber) or peripheral (uninjured myofiber) nuclei in each group. All scale bars indicate 100 μ m. + indicates difference compared to uninjured group. ++ indicates $P < 0.005$ ++++ indicates $P < 0.0001$. ns, not significant. Bars represent means $\pm$ standard deviation. Each point represents one animal.

Taken together, this data indicated that the pro-regenerative and anti-fibrotic effects of HCQ were not limited to TLR9 inhibition, and that muscle fibrosis in IR injury was also largely mediated by pathways independent of TLR9. We also concluded that muscle fibrosis was at least in part independent of NET formation in this model, as the increase in regeneration and reduction in fibrosis was accompanied by a non-significant decrease in NET formation.

2.5 HCQ Treatment inhibited fibro-adipogenic progenitor cell (FAP) proliferation

Fibro-adipogenic progenitor cells proliferate following injury and differentiate into myofibroblasts, which are responsible for ECM deposition and fibrotic deposition (21). Having shown the HCQ decreased ECM deposition and fibrosis following IR injury, we hypothesized that HCQ may be acting on FAPs. Full details of findings regarding FAP proliferation are reported in Edwards, et al (2020) (13). Briefly, by IF staining for FAPs (identified by expression of platelet derived growth factor receptor alpha, PDGFR α) and the proliferation marker Ki67 at 5 DPI revealed that HCQ treatment significantly decreased the proliferation in FAP cells, as shown by a decreased number of double-positive PDGFR α -Ki67 cells in the HCQ treated muscles compared to vehicle treated muscles (0.1 ± 0.3 vs 2.6 ± 1.6 vs 8.5 ± 4.7 ; uninjured, HCQ, vehicle, respectively; $n = 4-6$ /group, $P < .0001$, ordinary one-way ANOVA plus Tukey's test) (Fig. 9A-B). Additionally, we used a linear regression analysis to correlate total PDGFR α staining with total Ki67 staining and demonstrated that vehicle treated animals showed a significant linear correlation between PDGFR α and Ki67 stains ($P = 0.04$, Fig. 9E), which suggested that FAPs were the proliferating population. By contrast, there was poor concordance of FAP/proliferation correlation plots of the HCQ treated animals ($P = 0.17$ Fig 9D), which suggested that, while Ki67 staining was present, it did not correspond to increased PDGFR α expression.

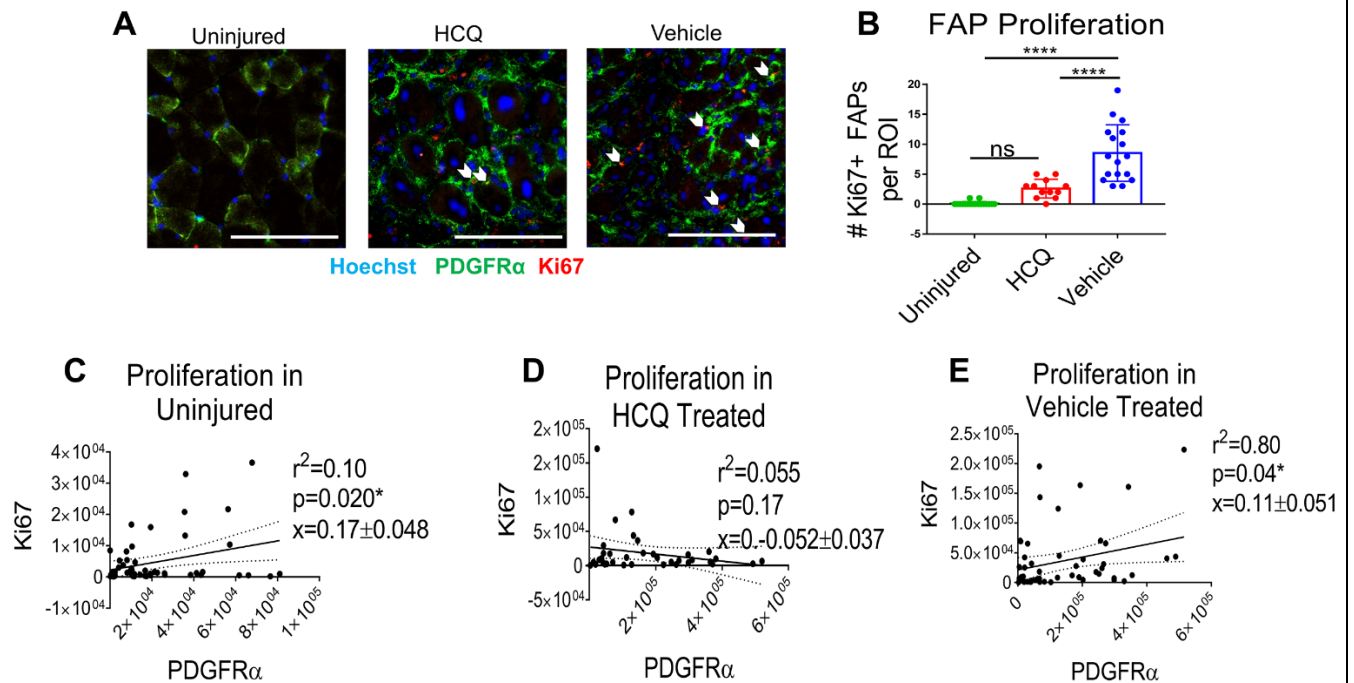


Figure 9: A) Immunofluorescent staining of FAPs co-stained with Ki67 (examples indicated by white arrowheads) in the TA at 5 DPI in C57BL/6 mice uninjured and treated with HCQ or vehicle. B) Quantification of number of FAPs expressing Ki67. HCQ treatment significantly reduces the number of FAPs expressing Ki67. Correlation curves of total PDGFRα+ and Ki67+ area in C) uninjured, D) HCQ treated, E) vehicle treated TA muscles. All scale bars indicate 100 μm *indicates $P < 0.05$; ****indicates $P < 0.0001$. Each point represents one ROI.

Following injury, FAPs may also differentiate to adipocytes (21). Oil Red O staining, which identifies lipid deposition, in the vehicle and HCQ treated TAs at 5 DPI identified very little lipid accumulation, indicating that FAPs were not driven towards adipogenesis following IR injury, nor did HCQ lead to adipocyte formation, as shown in **Fig. 10**.

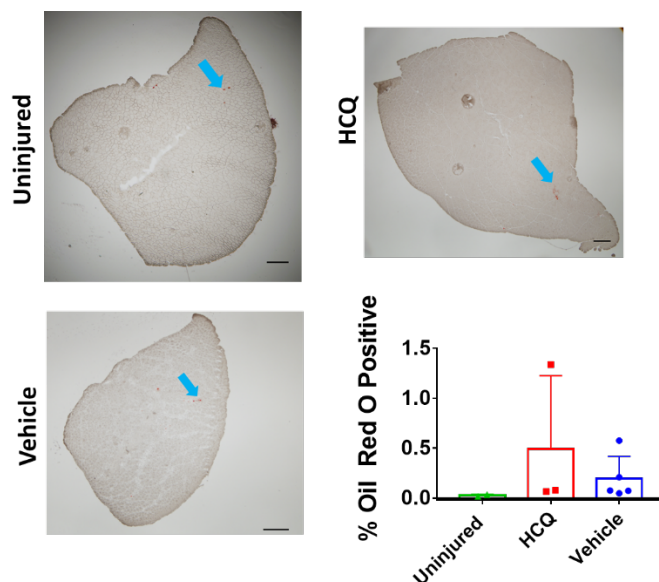


Figure 10: HCQ treatment does not induce adipocyte formation. Representative images of Oil Red O staining of C57BL/6 muscles at 5 DPI treated with vehicle, HCQ, or uninjured. Blue arrow indicates areas of positive staining. Quantification of percentage of muscle section stained positive for Oil Red O (area positive/total area of muscle section). No statistical difference between groups was found. Scale bars indicate 0.25 mm. $n=3-5$ per group; 3 slides per animal. Each dot represents average of one animal.

Taken together, this indicated that treatment with HCQ decreased proliferation in the FAP population at 5 DPI, which corresponded to a decrease in fibrosis at 7 DPI.

2.6 Treatment with HCQ inhibits ERK1/2 phosphorylation after IR injury

The role of ERK signaling following IR injury is not yet completely elucidated, but most studies to date show decreased ERK signaling and/or decreased ERK1/2 phosphorylation in the injured tissue to be beneficial for tissue survival and regeneration (22-27) and ERK signaling has also been implicated in fibrotic diseases (28-32). Therefore, we sought to investigate the role of *Mapk* expression and ERK1/2 signaling in the context of IR injury. By 7 DPI, at which point a difference in fibrotic deposition is seen, *Mapk1* and *Mapk3* expression is similar in whole muscle tissue treated with HCQ or with vehicle control (**Fig. 11 A-B**). Similarly, whole tissue protein analysis by western blot shows similar levels of ERK1/2 protein in tissues treated with HCQ or vehicle control at 7 DPI (**Fig. 11C**). However, significant differences are present in the levels of phosphorylated ERK1/2 (pERK1/2) at this time point (**Fig. 11D**, $p < 0.05$). Animals treated with HCQ show significantly lower ratios of pERK1/2 to ERK1/2 than the vehicle control treated mice. At 7 DPI, mice treated with HCQ also present with decreased muscle fibrosis, supporting the hypothesis that inhibition of ERK1/2 phosphorylation attenuates muscle fibrosis (13).

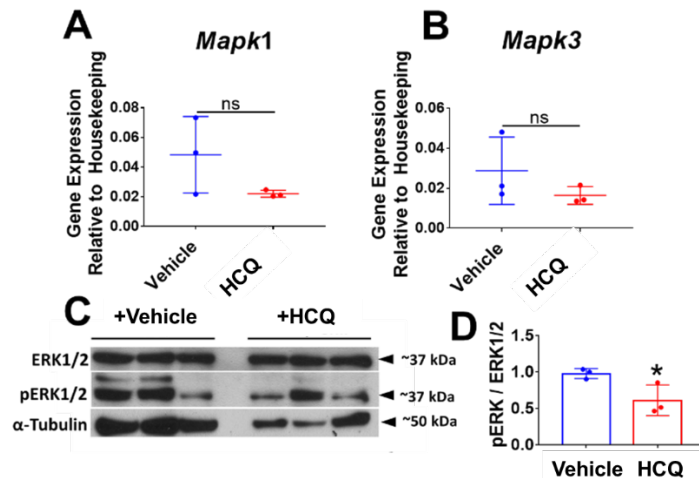


Figure 11: HCQ decreases ERK1/2 phosphorylation. At 7 DPI, A) *Mapk1* and B) *Mapk3* levels are not significantly different in whole muscle tissue treated with vehicle control (PBS) or HCQ. Gene expression calculated using qPCR and expressed relative to housekeeping gene. Unpaired t-test used for statistical analysis; $n=3/\text{group}$. C) Western blots of proteins ERK1/2, phosphorylated ERK1/2 (pERK1/2) and alpha-tubulin in whole muscle tissue at 7 days post IR injury in mice treated with vehicle control or HCQ. D) Ratio of pERK to ERK1/2 was significantly lower in animals treated with HCQ. Analyzed with unpaired t-test, $n=3$ per group, *denotes $p < 0.05$.

2.7 HCQ dosing does not lead to mitochondrial dysfunction in injured muscle

Although chloroquine-induced myopathy has been reported, this side effect is rare and reversible once the dosing regimen is stopped (33-35). In order to verify that 60 mg/kg HCQ for 7 days does not lead to mitochondrial dysfunction in the injured muscle, we performed a cytochrome c oxidase/succinate dehydrogenase (COX/SDH) histochemistry double stain to assess mitochondrial function. To quantitate, each myofibers was classified as COX positive/negative and SDH positive/negative. The percentage of COX positive/SDH negative myofibers and COX positive/SDH positive myofibers per total myofibers was determined for each image. The average percentage for each section was determined and used to determine the average per animal, reported as proportion of myofibers COX⁺/SDH⁻ or COX⁺/SDH⁺. We found that daily dosing of 60 mg/kg HCQ for 7 days did not lead to mitochondrial dysfunction in the injured muscle, as assessed by COX/SDH double staining, as shown in **Fig. 12**.

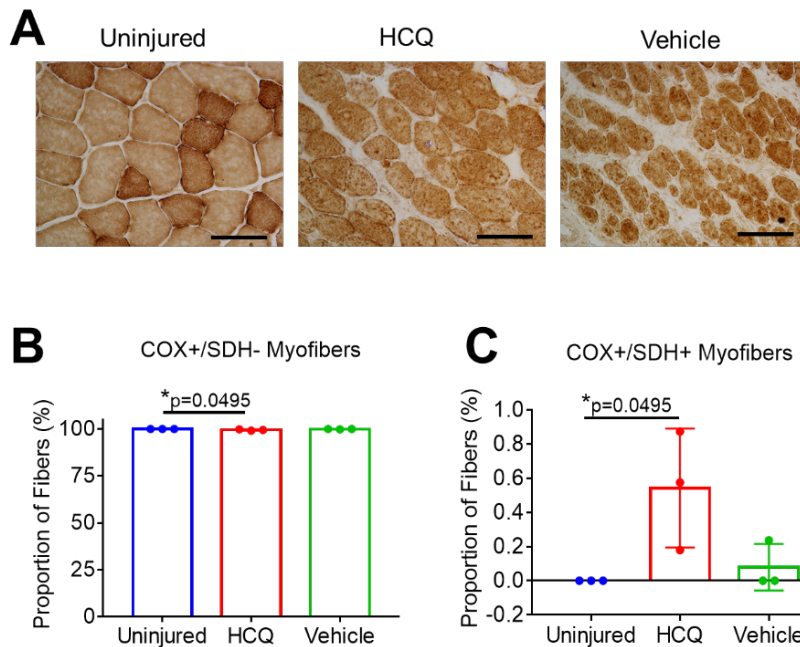


Figure 12: 7 day 60 mg/kg HCQ treatment does not result in mitochondrial myopathy. A) Representative images of C57BL/6 muscles at 7 DPI double stained with cytochrome c oxidase (COX) and succinate dehydrogenase (SDH). B-C) Quantification of proportion of myofibers positive for COX and/or SDH stain. n=3 animals/group. Each point represents one animal; average of three slides per animal. Difference between HCQ and vehicle treatment was not significant. * indicates $p < 0.05$. Scale bars indicate 100 μ m.

Key Findings or Accomplishments for Specific Aim 2:

In Specific Aim 2, we have identified FDA-approved hydroxychloroquine (Plaquenil) as a treatment to mitigate skeletal muscle fibrosis and increase muscle regeneration following IR injury. We identified that HCQ acts by decreasing proliferation in FAP cells, which leads to decreases collagen deposition. HCQ treatment also decreases ERK1/2 phosphorylation in the whole muscle tissue. We also identified that, HCQ (TLR7/8/9 inhibitor) does not significantly decrease NETs formation at 3 DPI. Similarly, TLR9^{-/-} mice did not show a decrease in NETs formation compared to wild type controls. Furthermore, TLR9^{-/-} mice and mice treated with TLR9 inhibitor ODN-2088 did not present with decreased muscle fibrosis, indicating that HCQ acts through mechanisms outside of TLR9 inhibition to promote regeneration and decreases skeletal muscle fibrosis.

References

- Gillani, S., Cao, J., Suzuki, T., and Hak, D.J., (2012) The effect of ischemia reperfusion injury on skeletal muscle. *Injury*. **43**(6): 670-5
- Oklu, R., Albadawi, H., Jones, J.E., Yoo, H.J., and Watkins, M.T., (2013) Reduced hind limb ischemia-reperfusion injury in Toll-like receptor-4 mutant mice is associated with decreased neutrophil extracellular traps. *J Vasc Surg*. **58**(6): 1627-36
- Albadawi, H., Oklu, R., Raacke Malley, R.E., O'Keefe, R.M., Uong, T.P., Cormier, N.R., and Watkins, M.T., (2016) Effect of DNase I treatment and neutrophil depletion on acute limb ischemia-reperfusion injury in mice. *J Vasc Surg*. **64**(2): 484-493
- Patel, R., Albadawi, H., Steudel, W., Hashmi, F.F., Kang, J., Yoo, H.J., and Watkins, M.T., (2012) Inhalation of carbon monoxide reduces skeletal muscle injury after hind limb ischemia-reperfusion injury in mice. *Am J Surg*. **203**(4): 488-95
- Volker Brinkmann, U.R., Christian Goosmann, Beatrix Fauler, Yvonne Uhlemann, David S. Weiss, Yvette Weinrauch, Arturo Zychlinsky, (2004) Neutrophil Extracellular Traps Kill Bacteria. *Science*. **303**
- Li, P., Li, M., Lindberg, M.R., Kennett, M.J., Xiong, N., and Wang, Y., (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med*. **207**(9): 1853-62
- Kusunoki, Y., Nakazawa, D., Shida, H., Hattanda, F., Miyoshi, A., Masuda, S., Nishio, S., Tomaru, U., Atsumi, T., and Ishizu, A., (2016) Peptidylarginine Deiminase Inhibitor Suppresses Neutrophil Extracellular Trap Formation and MPO-ANCA Production. *Front Immunol*. **7**: 227
- Lewis, H.D., Liddle, J., Coote, J.E., Atkinson, S.J., Barker, M.D., Bax, B.D., Bicker, K.L., Bingham, R.P., Campbell, M., Chen, Y.H., Chung, C.W., Craggs, P.D., Davis, R.P., Eberhard, D., Joberty, G., Lind, K.E., Locke, K., Maller, C., Martinod, K., Patten, C., Polyakova, O., Rise, C.E., Rudiger, M., Sheppard, R.J.,

- Slade, D.J., Thomas, P., Thorpe, J., Yao, G., Drewes, G., Wagner, D.D., Thompson, P.R., Prinjha, R.K., and Wilson, D.M., (2015) Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol.* **11**(3): 189-91
9. Yanming Wang, J.W., Joyce Sayegh, Young-Ho Lee, Julie R. Perlin, Lauriebeth Leonelli, Lakshmi S. Sonbuchner, Charles H. McDonald, Richard G. Cook, Yali Dou, Robert G. Roeder, Steven Clarke, Michael R. Stallcup, C. David Allis, Scott A. Coonrod, (2004) Human PAD4 Regulates Histone Arginine Methylation Levels via Demethylation. *Science.* **306**: 279-283
10. Knight, J.S., Zhao, W., Luo, W., Subramanian, V., O'Dell, A.A., Yalavarthi, S., Hodgins, J.B., Eitzman, D.T., Thompson, P.R., and Kaplan, M.J., (2013) Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest.* **123**(7): 2981-93
11. Willis, V.C., Gizinski, A.M., Banda, N.K., Causey, C.P., Knuckley, B., Cordova, K.N., Luo, Y., Levitt, B., Glogowska, M., Chandra, P., Kulik, L., Robinson, W.H., Arend, W.P., Thompson, P.R., and Holers, V.M., (2011) N-alpha-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. *J Immunol.* **186**(7): 4396-404
12. Moscarello, M.A., Lei, H., Mastronardi, F.G., Winer, S., Tsui, H., Li, Z., Ackerley, C., Zhang, L., Rajmakers, R., and Wood, D.D., (2013) Inhibition of peptidyl-arginine deiminases reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. *Dis Model Mech.* **6**(2): 467-78
13. Edwards, N., (2020) The Role of Neutrophil Extracellular Traps and TLR Signaling in Skeletal Muscle Ischemia Reperfusion Injury. *FASEB J*,
14. Delgado-Rizo, V., Martinez-Guzman, M.A., Iniguez-Gutierrez, L., Garcia-Orozco, A., Alvarado-Navarro, A., and Fafutis-Morris, M., (2017) Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. *Front Immunol.* **8**: 81
15. Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., Punaro, M., Baisch, J., Guiducci, C., Coffman, R.L., Barrat, F.J., Banchereau, J., and Pascual, V., (2011) Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med.* **3**(73): 73ra20
16. Agarwal, S., Loder, S.J., Cholok, D., Li, J., Bian, G., Yalavarthi, S., Li, S., Carson, W.F., Hwang, C., Marini, S., Pagani, C., Edwards, N., Delano, M.J., Standiford, T.J., Knight, J.S., Kunkel, S.L., Mishina, Y., Ward, P.A., and Levi, B., (2019) Disruption of Neutrophil Extracellular Traps (NETs) Links Mechanical Strain to Post-traumatic Inflammation. *Front Immunol.* **10**: 2148
17. Itagaki, K., Kaczmarek, E., Lee, Y.T., Tang, I.T., Isal, B., Adibnia, Y., Sandler, N., Grimm, M.J., Segal, B.H., Otterbein, L.E., and Hauser, C.J., (2015) Mitochondrial DNA released by trauma induces neutrophil extracellular traps. *PLoS One.* **10**(3): e0120549
18. Tang, T.T., Lv, L.L., Pan, M.M., Wen, Y., Wang, B., Li, Z.L., Wu, M., Wang, F.M., Crowley, S.D., and Liu, B.C., (2018) Hydroxychloroquine attenuates renal ischemia/reperfusion injury by inhibiting cathepsin mediated NLRP3 inflammasome activation. *Cell Death Dis.* **9**(3): 351
19. Bourke, L., McCormick, J., Taylor, V., Pericleous, C., Blanchet, B., Costedoat-Chalumeau, N., Stuckey, D., Lythgoe, M.F., Stephanou, A., and Ioannou, Y., (2015) Hydroxychloroquine Protects against Cardiac Ischaemia/Reperfusion Injury In Vivo via Enhancement of ERK1/2 Phosphorylation. *PLoS One.* **10**(12): e0143771
20. Fang, H., Liu, A., Dahmen, U., and Dirsch, O., (2013) Dual role of chloroquine in liver ischemia reperfusion injury: reduction of liver damage in early phase, but aggravation in late phase. *Cell Death Dis.* **4**: e694
21. Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., Yoneda, T., Segawa, M., Yamaguchi, M., Ogawa, R., Matev, M.M., Miyagoe-Suzuki, Y., Takeda, S., Tsujikawa, K., Tsuchida, K., Yamamoto, H., and Fukada, S., (2011) Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J Cell Sci.* **124**(Pt 21): 3654-64
22. Collier, J.B., Whitaker, R.M., Eblen, S.T., and Schnellmann, R.G., (2016) Rapid Renal Regulation of Peroxisome Proliferator-activated Receptor gamma Coactivator-1alpha by Extracellular Signal-Regulated Kinase 1/2 in Physiological and Pathological Conditions. *J Biol Chem.* **291**(52): 26850-26859
23. Okada, M., Yamane, M., Yamamoto, S., Otani, S., Miyoshi, K., Sugimoto, S., Matsukawa, A., Toyooka, S., Oto, T., and Miyoshi, S., (2018) SPRED2 deficiency may lead to lung ischemia-reperfusion injury via ERK1/2 signaling pathway activation. *Surg Today.* **48**(12): 1089-1095
24. Xu, S., Niu, P., Chen, K., Xia, Y., Yu, Q., Liu, N., Li, J., Li, S., Wu, L., Feng, J., Wang, W., Lu, X., Liu, T., Wang, F., Dai, W., Fan, X., Mo, W., Xu, L., and Guo, C., (2017) The liver protection of propylene glycol alginate sodium sulfate preconditioning against ischemia reperfusion injury: focusing MAPK pathway activity. *Sci Rep.* **7**(1): 15175
25. Zhang, J., Xia, J., Zhang, Y., Xiao, F., Wang, J., Gao, H., Liu, Y., Rong, S., Yao, Y., Xu, G., and Li, J., (2016) HMGB1-TLR4 signaling participates in renal ischemia reperfusion injury and could be attenuated by dexamethasone-mediated inhibition of the ERK/NF-kB pathway. *Am J Transl Res.* **8**(10): 4054-4067

26. Zhou, Q.L., Teng, F., Zhang, Y.S., Sun, Q., Cao, Y.X., and Meng, G.W., (2018) FPR1 gene silencing suppresses cardiomyocyte apoptosis and ventricular remodeling in rats with ischemia/reperfusion injury through the inhibition of MAPK signaling pathway. *Exp Cell Res.* **370**(2): 506-518
27. Zhu, B.L., Xie, C.L., Hu, N.N., Zhu, X.B., and Liu, C.F., (2018) Inhibiting of GRASP65 Phosphorylation by DL-3-N-Butylphthalide Protects against Cerebral Ischemia-Reperfusion Injury via ERK Signaling. *Behav Neurol.* **2018**: 5701719
28. Andrikopoulos, P., Kieswich, J., Pacheco, S., Nadarajah, L., Harwood, S.M., O'Riordan, C.E., Thiemermann, C., and Yaqoob, M.M., (2019) The MEK Inhibitor Trametinib Ameliorates Kidney Fibrosis by Suppressing ERK1/2 and mTORC1 Signaling. *J Am Soc Nephrol.* **30**(1): 33-49
29. Foglia, B., Cannito, S., Bocca, C., Parola, M., and Novo, E., (2019) ERK Pathway in Activated, Myofibroblast-Like, Hepatic Stellate Cells: A Critical Signaling Crossroad Sustaining Liver Fibrosis. *Int J Mol Sci.* **20**(11)
30. Kim, J., Kim, B., Kim, S.M., Yang, C.E., Song, S.Y., Lee, W.J., and Lee, J.H., (2019) Hypoxia-Induced Epithelial-To-Mesenchymal Transition Mediates Fibroblast Abnormalities via ERK Activation in Cutaneous Wound Healing. *Int J Mol Sci.* **20**(10)
31. Muchir, A., Kim, Y.J., Reilly, S.A., Wu, W., Choi, J.C., and Worman, H.J., (2013) Inhibition of extracellular signal-regulated kinase 1/2 signaling has beneficial effects on skeletal muscle in a mouse model of Emery-Dreifuss muscular dystrophy caused by lamin A/C gene mutation. *Skelet Muscle.* **3**(17): 1-10
32. Zhong, W., Shen, W.F., Ning, B.F., Hu, P.F., Lin, Y., Yue, H.Y., Yin, C., Hou, J.L., Chen, Y.X., Zhang, J.P., Zhang, X., and Xie, W.F., (2009) Inhibition of extracellular signal-regulated kinase 1 by adenovirus mediated small interfering RNA attenuates hepatic fibrosis in rats. *Hepatology.* **50**(5): 1524-36
33. Casado, E., Gratacos, J., Tolosa, C., Martinez, J.M., Ojanguren, I., Ariza, A., Real, J., Sanjuan, A., and Larrosa, M., (2006) Antimalarial myopathy: an underdiagnosed complication? Prospective longitudinal study of 119 patients. *Ann Rheum Dis.* **65**(3): 385-90
34. Avina-Zubieta, J.A., Johnson, E.S., Suarez-Almazor, M.E., and Russell, A.S., (1995) Incidence of Myopathy in Patients Treated with Antimalarials. A Report of Three Cases and a Review of the Literature. *Br J Rheumatol.* **34**
35. Khosa, S., Khanlou, N., Khosa, G.S., and Mishra, S.K., (2018) Hydroxychloroquine-induced autophagic vacuolar myopathy with mitochondrial abnormalities. *Neuropathology.* **38**(6): 646-652

What opportunities for training and professional development has the project provided?

This project has provided training in animal research and handling methods, laboratory techniques, data analysis, and project management skills for the post-doctoral fellows and research personnel that have worked on this project.

How were the results disseminated to communities of interest?

Data from this project was shared with colleagues in orthopaedic surgery, trauma surgery, immunology, cell and developmental biology, and other applicable fields through research presentations, research collaboration meetings, and joint laboratory meetings. This work has been published as an abstract at the Military Health Science Research Symposium 2020 annual meeting and in *The FASEB Journal* and *Journal of Immunology*. Work related to this project has also been submitted to the 2021 annual meeting of the Orthopaedic Research Society.

What do you plan to do during the next reporting period to accomplish the goals?

Not applicable/final report.

Describe the Regulatory Protocol and Activity Status (if applicable).

(a) Human Use Regulatory Protocols

TOTAL PROTOCOL(S): No human subject research will be performed to complete the Statement of Work.

(b) Use of *Human* Cadavers for Research Development Test & Evaluation (RDT&E), Education or Training

TOTAL ACTIVITIES: No RDT&E, education or training activities involving human cadavers will be performed to complete the Statement of Work (SOW).

ACTIVITIES: NA

(c) Animal Use Regulatory Protocols

TOTAL PROTOCOL(S): 1 animal use research protocols will be required to complete the Statement of Work

PROTOCOL (1 of 1 total):

IACUC Protocol Number: PRO00007909

Protocol PI: Levi

Site: University of Michigan

IACUC Title: **Study and Development of Therapeutic Option for the Treatment of Burn, Trauma, and Heterotopic Ossification**

ACURO Protocol Number: OR160105

Target required for clinical significance: 1

Target approved for clinical significance: 1

SUBMITTED TO AND APPROVED BY:

- submitted to IACUC for review: Nov 9, 2017
- approved by IACUC: Sept 20, 2017
- submitted to USAMRMC ACURO, July 19, 2017
- approved by USAMRMC ACURO, Nov 11, 2017

STATUS:

- Approved and appropriate with no needed revisions at this point

4. Impact:

What was the impact on the development of the principal discipline(s) of the project?

This project has provided knowledge about the role of neutrophils and neutrophil extracellular traps, PAD4 and TLR9 pathways in ischemia-reperfusion injury. We have also identified a promising treatment strategy to mitigate skeletal muscle fibrosis following IR injury which can be developed into a clinical treatment for soldiers and civilians at risk for IR injury.

What was the impact on other disciplines?

This project has provided knowledge which will be important for immunology and neutrophil biology, understanding neutrophil extracellular trap formation, and the role of NETs in injury. This project is also important for broader muscle injury and regeneration, and ischemia-reperfusion injury in other organs such as liver, kidney, heart and lungs.

What was the impact on technology transfer?

These findings may lead to use of FDA-approved Plaquenil (hydroxychloroquine) as a treatment to mitigate muscle fibrosis.

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

None

Actual or anticipated problems or delays and actions or plans to resolve them

None

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

6. Products

- "Novel FDA Approved Therapy to Mitigate Ischemia-Reperfusion Induced Fibrosis" *Abstract accepted at Military Health Science Research Symposium 2020* (Appendix A)
- "Novel Ultrasound Imaging Method for Early Identification of Combat Trauma-Induced Heterotopic Ossification" *Abstract accepted at Military Health Science Research Symposium 2020*
- Edwards NJ, Hwang C, Marini S, et al. The role of neutrophil extracellular traps and TLR signaling in skeletal muscle ischemia reperfusion injury. *The FASEB Journal*. 2020 *In Press* (Appendix B)
- Stepien DM, Hwang, C, Marini, S, et al. Tuning Macrophage Phenotype to Mitigate Skeletal Muscle Fibrosis. *J Immunology*. 2020 (204)8:2203-2215. DOI: 10.4049/jimmunol.1900814
- "High Frequency Ultrasound Imaging Detects Early Post Traumatic Heterotopic Ossification in Rodents" *Original research article in Preparation for peer reviewed journal submission*

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name: Benjamin Levi

Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Planning of experimentation.
Name:	Nicole Edwards
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1-No effort on project as she is being covered by a T32 training grant.
Contribution to Project:	Dr. Edwards has been the project lead; performed experimental planning, execution, and data analysis.
Name:	Kaetlin Vasquez
Project Role:	Animal and lab manager
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2.
Contribution to Project:	Kaetlin has managed the animal colony and coordinated logistical aspects of this project.
Name:	Karen Kessell
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Karen has worked on analysis of the IR model by performing analytics and histology.
Name:	Noelle Visser
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3
Contribution to Project:	Noelle has worked on analysis of the IR model by performing analytics and histology.
Name:	Peter Ward
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Dr. Ward has worked with Dr. Levi to help guide the project.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements:

Quad Charts:



PI: Benjamin Levi MD

Org: University of Michigan, Ann Arbor, MI Award Amount: \$500,000

Study/Product Aim(s)

The goals of this grant are to establish the efficacy of inhibition of neutrophil extracellular traps (NETs) to abrogate extremity trauma and ischemia-reperfusion soft tissue injury. Specifically, we will inhibit peptidyl arginine deiminase 4 (PAD4) and toll-like receptor 9 (TLR9) using FDA approved novel inhibitors to prevent acute hind limb injury in an animal model relevant to military and civilian extremity injuries.

Approach

Aim 1: To demonstrate that NETs exacerbate skeletal IR injury and to validate that PAD4 inhibition with GSK199 or Cl-Amidine prevents NETosis, endothelial cell damage and soft tissue injury after hind limb ischemic trauma without impaired wound healing.

Aim 2: To decrease endothelial cell dysfunction and to prevent progression of ischemia reperfusion soft tissue injury using FDA-approved TLR9 inhibitors to mitigate secondary NET propagation (NET induced NETosis).

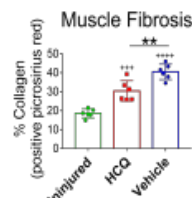
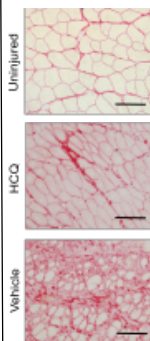


Figure 1: Plaquenil (HCQ) treatment decreases skeletal muscle fibrosis following IR injury. Picrosirius red stain showing collagen deposition in the IR injured tibialis anterior of C57BL/6 mice at 7 days post injury. Treatment with 60 mg/kg HCQ significantly decreases skeletal muscle fibrosis compared to vehicle control treatment. Scale bars indicate 100 μ m. ** indicates $P < 0.01$; $n = 6$ /group. Analyzed with ordinary one-way ANOVA plus Tukey's test. Bars indicate mean $\pm$ std dev. Each point represents one animal.

Timeline and Cost

Activities	CY	17	18	19
Validate PAD4 inhibitors (GSK199 and Cl-Amidine and TLR9 inhibitors (ODN-2088 and Plaquenil) to decrease endothelial cell dysfunction after IR injury.				
Defining the stages of endothelial cell dysfunction and muscle fibrosis (with histology, intravital microscopy, FACS and ultrasound imaging after IR injury.				
Analysis of muscle function after ischemia reperfusion injury treated with Cl-Amidine, GSK199, ODN-2088, Plaquenil or control.				
Requested Budget (500\$K)		\$100K	\$200K	\$200K

Goals/Milestones

CY17-18 Goals

- ☐ We will define the stages of ischemia reperfusion induced endothelial cell dysfunction and muscle fibrosis.
- ☐ We will use the acute hind limb ischemia mouse model to assess the effect of inhibition of NET induced endothelial cell dysfunction and inflammation *in vivo* using translational PAD4 inhibitors Cl-Amidine and GSK199 and TLR9 inhibitors ODN-2088 and Plaquenil.

CY18-19 Goals

- ☐ We will validate treatment with Cl-Amidine, GSK199, Plaquenil and ODN-2088 to decrease IR induced inflammation (flow cytometry and histology) and muscle fibrosis (SUSI, histology and functional assays). We will perform muscle function analysis by taking *in situ* measurements of whole muscle force contractile properties and gait analysis.

Comments/Challenges/Issues/Concerns

- N/A

Budget Expenditure to Date

Projected Expenditure: \$0.5M

Actual Expenditure: \$490,571

Novel FDA Approved Therapy to Mitigate Ischemia-Reperfusion Induced Fibrosis

N. Edwards, PhD; C. Hwang, BS; C. Pagani, BA; N. Visser, MS; A. Mei; P. Yu, BS; T. Davis, PhD, B. Levi, MD

Background and Objective Tissue damage from ischemia reperfusion (IR) injury occurs when blood supply is restored to the tissue following a period of ischemia, such as after a traumatic injury and extended tourniquet application following a battlefield extremity injury. While tourniquet application successfully achieves hemostasis, complications secondary to tourniquet use often arise (1). When blood supply is interrupted to skeletal muscle tissue, devastating fibrosis often results in the affected muscle, leading to loss of function and decreased range of motion (2,3). Although the mechanisms contributing to IR injury are complex and not completely elucidated, a vigorous neutrophil response is well-documented in the initial phase, which may be excessive and ultimately deleterious (4). Neutrophils at the injury site may also produce neutrophil extracellular traps (NETs), web-like structures of DNA and antimicrobial peptides that are extruded into the tissue as part of the innate immune response. While important in moderation, excess NET production may exacerbate tissue damage after traumatic injuries (5,6). Here, we use a mouse model of hind limb IR injury to investigate the role of neutrophils and NETs following IR injury and study methods for modulation of the neutrophil and NET response in order to mitigate skeletal muscle fibrosis. We hypothesized that FDA approved hydroxychloroquine (HCQ, Plaquenil®), which modulates the NETosis pathway through toll-like receptor 9, will mitigate skeletal muscle fibrosis following IR injury.

Methods Skeletal muscle fibrosis in the tibialis anterior (TA) muscle was induced in a mouse model of IR injury by clamping the femoral artery and vein immediately distal to the inguinal ligament for three hours, followed by release of the clamp and tissue reperfusion until the indicated time points. Cl-Amidine (10 mg/kg), HCQ (60 mg/kg) or their respective vehicle controls were administered once daily starting on the day of injury via intraperitoneal injection. The TA was harvested, histological sections prepared, and NETs investigated at 1, 3, 5, and 7 days post injury (DPI) using immunofluorescence staining of myeloperoxidase, citrullinated histone 3, and nuclei. Co-staining of nuclei and myeloperoxidase, myeloperoxidase and citrullinated histone 3, and/or a classic web-shaped deposition of nuclear material in the tissue were considered NETs. Fibrosis was quantified using picrosirius red staining on frozen TA sections. ImageJ was used to quantify the area of positive picrosirius red, indicating collagen deposition, and normalized by the total area of the muscle section in order to determine a percent muscle fibrosis. Myovision software was used to calculate myofiber cross sectional area (CSA) and Feret diameter based on laminin staining of regenerated myofibers by immunofluorescence.

Results NET formation was detected in the TA muscle at 1, 3, and 5-DPI and had largely been resolved by 7-DPI, as shown by immunofluorescence staining for myeloperoxidase, citrullinated histone 3, and nuclei, as described previously ($n \geq 3$ per time point). Fibrosis in the TA was quantified at 7-DPI in mice treated with the NET inhibitor Cl-Amidine or vehicle control. While NETs were significantly decreased at 3-DPI in the Cl-Amidine treated mice (NETs per 63x region of interest, average $\pm$ SD; Cl-Amidine: 3.9 ± 2.7 , vehicle control: 24.1 ± 11.7 , $p < 0.05$, $n = 3$ per group), fibrosis was not significantly changed at 7-DPI ($50.6\% \pm 5.5$ vs $47.7\% \pm 11.1$; Cl-Amidine, vehicle control, respectively, mean $\pm$ SD $p = 0.8$; $n \geq 4$ per group). Therefore, this finding indicates muscle fibrosis is not mediated by NETs formed via the PAD4 (peptidyl arginine deiminase 4) mechanism through which Cl-Amidine acts. NET propagation has been shown to be mediated through the toll-like receptor 9 (TLR9) pathway (6). In order to investigate this pathway, we administered TLR9 inhibitor HCQ following IR injury. At 3-DPI, NET formation was not significantly different in the HCQ vs vehicle control treated groups (NETs per 63x region of interest, average $\pm$ SD, HCQ: 4.5 ± 1.6 ; vehicle control: 7.4 ± 3.2 ; $p = 0.0705$; $n = 6$ per group), however by 7-DPI mice treated with HCQ presented with decreased muscle fibrosis

compared to vehicle control (HCQ: $30.4\% \pm 5.4$; vehicle control: $40.5\% \pm 4.0$, $p=0.0019$ $n=6$ per group). Treatment with HCQ also increased muscle regeneration. HCQ treated muscles presented with a decreased frequency of myofibers with small CSA ($p<0.0001$) and small Feret diameters ($p<0.0001$) at 5-DPI.

Conclusions Administration of HCQ following IR injury decreased skeletal muscle fibrosis and increased muscle regeneration. Vigorous NET formation was observed in the skeletal muscle after IR injury. Treatment with PAD4 inhibitor Cl-Amidine did decrease NETs at 3-DPI, however did not translate to a decrease in muscle fibrosis at 7-DPI compared to the vehicle control. These findings indicate that skeletal muscle fibrosis after IR injury is independent PAD4 mediated NET formation, and while the TLR9 inhibitor HCQ did decrease fibrosis, a significant decrease in NET formation was not present with HCQ treatment, indicating that NET formation following IR injury is, at least in part, TLR9 independent. Further investigation will focus on the mechanism of action of HCQ in muscle fibrosis. We have shown that treatment with FDA-approved hydroxychloroquine leads to decreased muscle fibrosis and increased myofiber regeneration following IR injury, suggesting hydroxychloroquine may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury.

Learning Objectives 1) Characterize the fibrotic response in skeletal muscle following ischemia reperfusion injury. 2) Quantify NET formation in the skeletal muscle following ischemia reperfusion injury. 3) Decrease skeletal muscle fibrosis following ischemia reperfusion injury by administration of hydroxychloroquine following injury.

References 1) Beekley, AC et al. (2008). *J. Trauma* **64**: S28-S37. 2) Cholok, D et al.(2016). *J Trauma Acute Care Surg.* **82**(1): 174-184. 3) Gillani, S et al. (2012). *Injury* **43**(6): 670-675. 4) Honda, M et al. *Transplantation* **95**(4): 551-558. 5) McIlroy DJ et al (2014) *J. Crit. Care* 29(6): 1133.e1-5. 6) Agarwal S. et al. (2019) *Front Immunol.* **10**:2148.

Abstract Disclaimer Contents of this publication are sole responsibility of author(s) and do not necessarily reflect views, opinions or policies of Uniformed Services University of the Health Sciences, Dept. of Defense, Departments of the Army, Navy or Air Force. Mention of trade names, commercial products or organizations does not imply endorsement by US Government.

The Role of Neutrophil Extracellular Traps and TLR Signaling in Skeletal Muscle Ischemia Reperfusion Injury

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Running Title: NETs in Ischemia Reperfusion Muscle Fibrosis

Nonstandard Abbreviations

COX: cytochrome oxidase

Ctx: Cardiotoxin

DPI: Days Post Injury

eMHC: Embryonic Myosin Heavy Chain

ERK 1/2: Extracellular signal-regulated kinases 1/2

FAP: Fibroadipogenic progenitor cells

H3Cit: Citrullinated Histone H3

HCQ: Hydroxychloroquine

IL-10: Interleukin 10

IR: Ischemia Reperfusion

Mapk: Mitogen-activated kinase gene

MPO: Myeloperoxidase

NET(s): Neutrophil Extracellular Trap(s)

PAD4: Peptidylarginine deiminase 4

SDH: succinate dehydrogenase

TA: Tibialis Anterior

t-SNE: *t*-distributed stochastic neighbor embedding

TLR: Toll-like receptor

TNF α : Tumor Necrosis Factor alpha

Abstract

Ischemia reperfusion (IR) injury results in devastating skeletal muscle fibrosis. Here, we recapitulate this injury with a mouse model of hindlimb IR injury which leads to skeletal muscle fibrosis. Injury resulted in extensive immune infiltration with robust neutrophil extracellular trap (NET) formation in the skeletal muscle, however direct targeting of NETs via the peptidylarginine deiminase 4 (PAD4) mechanism was insufficient to reduce muscle fibrosis. Circulating levels of IL-10 and TNF α were significantly elevated post injury, indicating toll-like receptor (TLR) signaling may be involved in muscle injury. Administration of hydroxychloroquine (HCQ), a small molecule inhibitor of TLR7/8/9, following injury reduced NET formation, IL-10, and TNF α levels and ultimately mitigated muscle fibrosis and improved myofiber regeneration following IR injury. HCQ treatment decreased fibroadipogenic progenitor cell proliferation and partially inhibited ERK1/2 phosphorylation in the injured tissue, suggesting it may act through a combination of TLR7/8/9 and ERK signaling mechanisms. We demonstrate that treatment with FDA-approved HCQ leads to decreased muscle fibrosis and increased myofiber regeneration following IR injury, suggesting short-term HCQ treatment may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury.

Key words: muscle fibrosis, inflammation, extracellular signal-regulated kinases (ERK) 1/2, hydroxychloroquine

Introduction

Ischemia reperfusion (IR) tissue injury can occur after any situation in which the blood supply to a tissue is profoundly interrupted resulting in microcirculatory hypo-perfusion and hypoxia. Anaerobic metabolism leads to accumulation of lactic acid, decrease of pH in tissues (metabolic acidosis), mitochondrial dysfunction, vasoactive agents, generation of reactive oxygen species, oxidative stress, endothelial activation, aberrant local inflammatory responses, organelle damage and ultimately cell death (1, 2). Ischemia reperfusion injury occurs clinically both in controlled surgical settings such as organ transplantation and free flap procedures as well as during traumatic events, such as battlefield blast injuries involving emergency tourniquet usage to control hemorrhage. In the skeletal muscle, IR injury translates into inflammation, tissue edema, muscle fibrosis, and/or necrosis, ultimately leading to decreased range of motion or complete loss of function (3). Therefore, determining the precise mechanism by which IR injury occurs and discover treatments to minimize the long-term consequences is of utmost importance.

The resultant tissue damage occurs secondary to tissue hypoxia and paradoxically from the reperfusion of reoxygenated blood into the ischemic tissue often referred to as hypoxic re-oxygenation injury (2, 4). One cause of IR induced tissue injury is due to an excessive innate local and systemic inflammatory immune response. Perfused leukocytes encounter released intercellular damage-associated molecular patterns (DAMPs), adhesion molecules, chemokines, glycosaminoglycans (GAGs), metalloproteinase, and extracellular calcium and adenosine triphosphate (ATP) released by necrotic cells, which induce the activation of many toll-like receptor (TLR) pathways to promote an inflammatory response (1, 5-7). Neutrophils comprise a substantial portion of the amplifier cell population in the initial immune response, and their involvement in IR injury is hypothesized to be, in part, due to the formation of neutrophil extracellular traps (NETs) containing web-like extrusions of nuclear DNA, citrullinated histones, and antimicrobial peptides released into the tissue by activated neutrophils (8-10). NETs have been implicated in a wide variety of diseases, notably autoimmune diseases such as lupus (11-13) and rheumatoid arthritis (14, 15). Additionally, NETs have also been shown to form following traumatic injury both systemically and at the site of injury (16-21), including in models of ischemic injury in vital organs (5, 6, 18, 22).

NETs have been shown to form through several other independent pathways in addition to the well-characterized peptidylarginine deiminase 4 (PAD4) dependent pathway (23). Recent reports suggest that NET formation also occurs when DNA and RNA stimulate toll-like receptors (TLR) on polymorphonuclear neutrophils in a process termed self-propagation or secondary NETosis (17, 24). TLR 7/8 and 9 are pattern recognition receptors expressed on cells of the innate immune system, which are classically believed to recognize pathogenic RNA and DNA, respectively. However, growing evidence suggests that aberrant

activation of these TLRs with self DNA and/or RNA occur when free nucleic material is present in the tissue, such as in autoimmune diseases, viral infections, and following traumatic injuries (17, 25-31).

Small molecule inhibitors of TLR7/8/9, such as hydroxychloroquine (HCQ, Plaquenil®), were developed initially as anti-malarial agents, but have shown to be efficacious against lupus, an autoimmune disease characterized by excess and impaired degradation of NETs (13, 24, 32-34). HCQ has also been shown to be an effective antiviral agent against RNA viruses including dengue virus and retroviruses (35) by de-acidifying endosomes required for endosome-mediated viral entry during TLR7/8/9 activation.

IR injury also leads to abnormal expression of extracellular signal-regulated kinase 1/2 (ERK1/2), however, the precise role of ERK1/2 signaling in IR injury remains controversial. Mitogen-activated protein kinase (*Mapk*) genes, such as *Mapk3* (*Erk1*) and *Mapk1* (*Erk2*), have been found to be dysregulated in multiple pathological states, including fibrotic diseases. Studies have shown inhibition of ERK1/2 signaling has been shown to attenuate fibrosis by minimizing extracellular matrix deposition in several mouse models, including hepatic IR injury (36) and kidney fibrosis (37). Furthermore, inhibition of *Mapk* genes and decreased phosphorylation of ERK1/2 have protective effects in multiple organ systems by decreasing apoptosis and increasing cellular turnover (38-43). In contrast, contradicting reports have shown increased activation of the same ERK pathways provide protection (44-46). These differences suggest ERK signaling may vary depending on pathologic state/condition, thus the precise role in IR injury remains to be elucidated.

Given that excess nuclear material is observed following traumatic injury and may increase NET formation alone and through self-propagation by TLR7/8/9 signaling, we investigated the role of neutrophil signaling and NET formation in the fibrotic response of skeletal muscle in a murine IR injury model. We next hypothesized that modulation of TLR7/8/9 using HCQ, a small molecule inhibitor to TLR7/8/9, may lead to decreased tissue injury and skeletal muscle fibrosis following IR injury by interfering with secondary self-propagated NETosis mediated by TLR7/8/9 activation. More broadly, given the role of ERK1/2 in both cellular apoptosis following IR injury and in fibrotic diseases, we also evaluated the effects of HCQ treatment on *Erk1* and *Erk2* gene expression and ERK1/2 signaling following skeletal muscle IR injury. Coupling this with the known importance of neutrophils and the immune response following IR injury, we hypothesized that a combination therapy of TLR7/8/9 inhibition to target NETosis and inhibition of ERK1/2 activation would prevent skeletal muscle fibrosis following IR injury.

Here, we provide evidence that following IR injury in skeletal muscle, the initial immune response drives substantial NET formation in the injured muscle tissue that results in muscle fibrosis. We also show that HCQ treatment both inhibits proliferation of fibroadipogenic progenitor cells (FAPs), the population responsible for

fibrotic deposition (47), decreases phosphorylation of ERK1/2, and decreases NET formation. We find that administration of HCQ mitigates muscle fibrosis caused by IR injury through modulation of the initial neutrophil response, FAP proliferation, and ERK1/2 signaling.

Materials and Methods

Animals

Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; stock no. 000664). PAD4^{-/-} mice were crossed 10 generations onto a C57BL/6 background (48). Matching background strains were used as controls for all transgenic experiments. All mice were 6-9 weeks of age at time of injury. Adult male (11-12 week-old, 350-450g) pathogen free Sprague Dawley rats were obtained from Taconic Biosciences (Germantown, NY, USA). Rats were housed for a minimum of 7 days for acclimatization and quarantine purposes in the vivarium located at the Walter Reed Army Institute of Research (Silver Spring, MD, USA). Animals were housed and maintained under standard conditions: 72 ± 4 °F, with a 12 hr light/dark cycle and no diet restrictions. All experiments and animal care procedures were approved by the University of Michigan (mouse protocol PRO 7930) and the Naval Medical Research Center and Walter Reed Army Institute of Research (protocol 17-OUMD-31S) institutional animal care and use committees. All activities were conducted in accordance with all applicable regulations.

Hindlimb Ischemia Reperfusion Injury in the Mouse Model

Mice received pre-operative 0.06 mg/kg buprenorphine subcutaneously as an analgesic. Mice were anesthetized with 1-3% isoflurane inhalation. A skin incision was made in the left groin and the groin fat pad was retracted to expose the femoral artery, vein, and nerve. The femoral artery and vein were separated from the nerve and an Ackland clamp was placed immediately distal to the inguinal ligament and proximal to the lateral circumflex femoral artery to induce acute hindlimb ischemia of the left lower limb. Mice remained under 2% isoflurane as left hindlimbs were subjected to 3 hr of ischemia. The clamp was removed and the hindlimb was reperfused until euthanasia at indicated time points. For mice with IR+cardiotoxin (IR+ctx) injury, 10 µL of 3 mg/ml cardiotoxin was injected into the left tibialis anterior muscle immediately after clamp removal. Ctx (*Naja pallida*, Sigma-Aldrich, St. Louis, MO, USA; cat. no. 217503) was dissolved in sterile phosphate buffered saline (PBS). Buprenorphine was administered at 0.06 mg/kg subcutaneously as an analgesic post-operatively and repeated at 12 and 24 hr.

Hindlimb Ischemia Reperfusion Injury in the Rat Model

Rats were administered prophylactic analgesia (Buprenex, 0.05 mg/kg and 1.2 mg/kg buprenorphine SR) via subcutaneous injection prior to injury and repeated at 48-72 hr, with further doses given as indicated based on animal assessment. Rats were anesthetized by intraperitoneal injection (IP; ketamine 80mg/kg and xylazine

10mg/kg). Additional boluses of ketamine and/or xylazine (10-20/1-3mg/kg) were administered to maintain a plane of general anesthesia. A pneumatic tourniquet (UDC1.6™, Hokanson, WA, USA) was used to interrupt blood flow. Limbs were elevated to reduce venous pooling and tourniquet was applied to the proximal aspect of the lower limb. The tourniquet was inflated and maintained at 280-300 mmHg pressure for 150 min.

Test Article Preparation and Dosing

Hydroxychloroquine sulfate (HCQ) (Cayman Chemical Ann Arbor, MI, USA, item no. 17911, CAS 747-36-4), was diluted to 15 mg/ml in sterile PBS and delivered via IP injection at 60 mg/kg daily, starting on the day of injury until euthanasia. A total of 100 µl of sterile PBS was delivered as the vehicle control (vehicle) via IP injection. Cl-amidine (Cayman Chemical, item no. 10599, CAS 1373232-26-8) was diluted to 50 mg/ml in dimethylsulfoxide as a stock solution. The stock solution was diluted to 2 mg/ml in sterile PBS and 10 mg/kg Cl-amidine was delivered via IP injection daily starting on the day of injury until euthanasia. A total of 150 µl of 4% dimethylsulfoxide in sterile PBS was delivered as the vehicle control through IP injection.

Tissue Harvesting and Histology/Immunohistochemistry

Tibialis anterior (TA) muscles were dissected away from the bone and immediately embedded and frozen in optimal cutting temperature (OCT) compound. Contralateral uninjured TA muscles were used as uninjured control samples. Samples were stored in OCT blocks at -80°C until sectioning. Sections were cut at 7-10 µm thick on a Leica 3050S cryostat, mounted on charged slides, and stored at -80°C until analysis. Standard H&E and picrosirius red protocols were followed for histological staining. For studies involving Cl-amidine treatment, 70% ethanol was used as a fixative prior to staining, for all other studies, Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) was used as a fixative. For immunofluorescent stains, samples were blocked and permeabilized with 2% serum, 1% BSA, 0.1% fish skin gelatin, 0.001% Triton X-100, and 0.0005% Tween 20. Primary antibodies (**Supp. Table 1**) were applied and incubated overnight at 4°C. Fluorescently conjugated secondary antibodies were added and then counterstained with Hoechst. Fluorescent samples were imaged using confocal fluorescence microscopy using a Leica SPX8 confocal microscope with 20x objective. All exposure times were optimized during initial imaging and kept constant for all subsequent imaging. Standard bright-field microscopy imaging was performed using a 4x, 20x, or 40x air objective. Appropriate primary antibody negative controls were run simultaneously with each tested sample.

NET Quantification

Sections (10 µm) of flash frozen TA muscle were stained with antibodies for citrullinated histone H3 (H3Cit), myeloperoxidase-conjugated with FITC (MPO-FITC), and Hoechst in concentrations described in **Supp. Table 1**. Three high powered representative images (63x resolution) were taken in similar areas of the muscle on one muscle section per animal using a Leica SPX8 confocal microscope. The number of NETs per region of interest

(ROI) were quantified by trained personnel and an average number of NETs per ROI was obtained per animal. NETs were considered co-localization of MPO-Hoechst, MPO-H3Cit-Hoechst, and/or classic web-like morphology of Hoechst and/or MPO.

Muscle Fibrosis Analysis

ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to calculate the percentage area of collagen based on staining with picrosirius red. The thresholding tool was used to create a mask of the area of positive staining, and this area was divided by the total area of the sample analyzed. The Huang method for thresholding was used for all images, and thresholding and analysis methods used were constant for every sample analyzed (49). Six non-overlapping images at 40x magnification were taken on one section for each animal. The average positive staining for these images was used as the percent fibrotic area for each animal. Sections from corresponding regions of the muscle were used for every analysis.

Cross Sectional Area Calculations

TA muscle sections (10 μ m, flash frozen) were stained with Hoechst 33342, eMHC and laminin fluorescently labeled antibodies. Myovision muscle analysis software (University of Kentucky, Lexington, KY, USA) (50) was used to detect muscle fibers and calculate average myofiber cross-sectional area and minimum Feret diameter in three separate, non-contiguous fields of regenerating muscle at 20x in 4-6 mice per group.

Centralized Nuclei Quantifications

TA muscle sections (10 μ m, flash frozen) were stained with hematoxylin and eosin (H&E) using standard protocols. Six non-overlapping images at 40x magnification were taken using bright field microscopy on three sections for each animal. One section was from the proximal, middle, and distal third. Nuclei within the myofiber were identified as either central or peripheral by trained personnel. The number of myofibers in each group was divided by the total number of myofibers per image to obtain the percentage of myofibers with centralized or peripheral nuclei. The average percentage for each section was determined and used to calculate the average per animal, which is reported as % Centralized Nuclei or % Peripheral Nuclei.

Cytochrome C Oxidase/Succinate Dehydrogenase (COX/SDH) Histochemistry and Quantification

COX/SDH staining was performed according to Ross (51). Briefly, slides were incubated in a solution of 1X 3,3'-Diaminobenzidine (Sigma-Aldrich; cat. no. D3939), 100 μ M cytochrome c (Sigma-Aldrich; cat. no. C2037) in PBS with 2 μ g bovine catalase (Sigma-Aldrich; cat. no. C9322) for 40 minutes at 37°C then washed with PBS. Next, slides were incubated with 1.5 mM nitroblue tetrazolium (Sigma-Aldrich; cat. no. N6876), 130 mM sodium succinate (Sigma-Aldrich; cat. no. 818601), 0.2 mM phenazine methosulfate (Sigma-Aldrich; cat. no. P9625), and 1.0 mM sodium azide (Sigma-Aldrich; cat. no. S8032) in PBS for 40 minutes at 37°C. Slides were washed, mounted and imaged with standard bright field microscopy. Six non-overlapping images at 40x

magnification were taken using bright field microscopy on three sections for each animal. One section was from the proximal, middle, and distal third. To quantify, each myofiber was classified as COX positive/negative and SDH positive/negative. The percentage of COX positive/SDH negative myofibers and COX positive/SDH positive myofibers per total myofibers was determined for each image. The average percentage for each section was determined and used to determine the average per animal, reported as proportion of myofibers COX+/SDH- or COX+/SDH+.

PDGFR α -Ki67 Staining and Quantification

Sections (10 μ m) of flash frozen TA muscle were stained with antibodies for platelet derived growth factor alpha (PDGFR α), Ki67, and Hoechst in concentrations described in **Supp. Table 1**. Three high powered representative images (63x magnification) were taken in similar areas of the muscle on three muscle sections per animal (one from each of the proximal, middle, and distal third of the muscle) using a Leica SPX8 confocal microscope. n=4-6 animals per group. The number of cells expressing co-localized PDGFR α and Ki67 were manually identified and counted per region of interest (ROI) by trained personnel. Total PDGFR α and Ki67 quantifications were determined with ImageJ to threshold positive area with the Yen thresholding method to determine total positive staining, which is reported in pixels. A linear regression analysis was used to correlate total PDGFR α and total Ki67 staining. In order to accurately correlate the amount of PDGFR α to the corresponding Ki67 expression in the same ROI, ROIs were evaluated and plotted individually in analyses.

Oil Red O Staining and Quantification

TA muscle sections (10 μ m, flash frozen) were fixed in in 4% paraformaldehyde and stained in Oil Red O solution (3 mg/mL Oil Red O; Sigma-Aldrich; CAS 1320-06-5; in 60% isopropyl alcohol) for 15 min. Slides were rinsed in tap water and mounted with an aqueous mountant. Images of the entire muscle section from corresponding locations in the TA were taken at 4x magnification using bright field microscopy. One section from the proximal, middle, and distal third of the TA were analyzed, to total three sections per animal. n=2-5 animals per group. ImageJ was used to calculate the percentage area of positive Oil Red O staining. The image was transformed to YIQ color spectrum, the 'I' spectrum was converted to an 8-bit image, and the MaxEntropy threshold was used to quantify the area stained positively for Oil Red O. The positive area was divided by the total area of the sample to yield the % Oil Red O positive. The mean of three sections per animal was reported as one point per animal.

Western Blots

TA tissue was sonicated and lysed with RIPA buffer lysis system (sc-24948A, Santa Cruz Biotechnology, Dallas, TX, USA) and exposed to three freeze-thaw cycles. Samples were assessed for total protein using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, cat no. 23225). Proteins from lysate were separated in

NuPAGE 4-12% bis-tris gel (ThermoFisher Scientific, cat no. NP0335BOX) with NuPAGE SDS running buffer (ThermoFisher Scientific, cat no. NP0001). Protein was transferred to PVDF membrane (ThermoFisher Scientific, cat no. IPVH00010) in NuPAGE transfer buffer (ThermoFisher Scientific, cat no. NP0006). Membrane was washed in wash buffer (1x Tris buffered saline-0.05% Tween-20) and blocked for 1 hr at room temperature with 5% milk protein in wash buffer. Membranes were incubated with primary antibody (**Supp. Table 1**) diluted in 5% BSA in wash buffer. Appropriate HRP-linked secondary was diluted in block solution. Membranes were developed and ImageJ software was used for quantification.

Cytokine Assays

For mouse cytokine analysis, blood samples were collected at 3 days post injury (DPI) in EDTA tubes from mice treated with vehicle or HCQ (n=3/group). Plasma was stored at -80°C until analysis without additional freeze-thaw cycles. On the day of assay, samples were diluted 1:1 with PBS and assessed using a mouse multi-spot 96-well 10-plex pro-inflammatory assay kit for mice (Mesoscale Diagnostics, Rockville, MD, USA) according to the manufacturer's guidelines. Plates were read and analyzed using the MSD Sector S600 Instrument and MSD Workbench Software version 4.0 (Mesoscale Diagnostics). A standard curve was generated to determine analyte concentration. For rat cytokine analysis, cytokine expression included here was determined in serum samples within a multiplex assay. Bio-Plex Pro™ Rat Cytokine 23-Plex assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA; cat# 12005641,) were performed on a Bio-Plex 200 Luminex system with high throughput fluidics (Bio-Rad Laboratories, Inc., cat# 171000205) which had undergone both validation (Bio-Plex Validation Kit 4.0, Bio-Rad Laboratories, Inc.) and daily calibration prior to use (Bio-Plex Calibration Kit, Bio-Rad Laboratories, Inc.) to standardize signal output and sensitivity between runs. Assay working ranges were determined using Bio-Plex Manager 6.1 software (Bio-Rad Laboratories, Inc.) and used for the automatic identification and removal of outliers and optimization of 5-PL standard curves to interpolate the concentrations of analytes in the unknown samples.

RNA Isolation and QRT-PCR

TA muscles were harvested at 7 days post IR injury from mice treated with vehicle or HCQ (n=3/group). Samples were flash frozen and stored at -80°C. Total RNA was isolated using ceramic bead homogenization and the RNeasy mini kit (Qiagen, Venlo, Netherlands). First strand cDNA was generated from 1 µg total RNA using the iScriptAdvanced cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). A custom low-density quantitative reverse transcription polymerase chain reaction array (qRT-PCR; Bio-Rad). The QuantStudio 7 Flex Real-Time PCR system (ThermoFisher Scientific) was used to run the custom arrays. Gene expression levels were calculated as normalized expression relative to housekeeping gene (*Rplp0*; dCt) or fold change gene expression (normalized value relative to vehicle treatment value; ddCt).

Single-cell RNA Sequencing using 10x Genomics

Single-cell tissue preparation and analysis was performed as previously described (52). Briefly, baseline uninjured muscle (day 0) and post-surgery day 3 TA muscles were harvested from six IR+ctx mice and eight uninjured C57BL/6 mice. 3 lanes of IR+ctx (n = 2 pooled mice per lane) and 2 lanes of uninjured (n = 4 pooled mice per lane) was used to maximize cell return. Harvested TAs were digested under agitation in 0.3% Type 1 Collagenase and 0.4% Dispase II (Life Technologies, Carlsbad, CA, USA. cat no 17100017 and 17105041) in RPMI 1640 medium for 45 min at 37°C. Cells were filtered, washed, and resuspended at ~1000 cells/ μ L. Cell viability was assessed and only samples with 85% or greater viability were used for sequencing. Single-cell 39 library generation was performed on the 10x Genomics Chromium Controller following the manufacturer's protocol for the v2 reagent kit (10x Genomics, Pleasanton, CA, USA). Single-cell gel bead bead-in-emulsions (GEMs) were generated, reverse transcription was performed, and the Post GEM-RT product was cleaned using DynaBeads MyOne Silane beads (ThermoFisher Scientific). cDNA was amplified, quantified and enzymatically fragmented and size selected for optimization using SPRIselect beads (Beckman Coulter, Brea, CA, USA). Double-sided SPRI bead cleanup was performed after end repair and A-tailing, and single-sided cleanup is done after adapter ligation. Indexes were added during PCR amplification, and a final double-sided SPRI cleanup was performed. During PCR amplification, indexes were added and double-sided SPRI was performed for final cleanup. Libraries were quantified by Kapa qPCR for Illumina Adapters (Roche) and size was determined by Agilent tapestation D1000 tapes. Read 1 primer sequence are added to the molecules during GEM incubation. P5, P7, and sample index and read 2 primer sequence are added during library construction via end repair, A-tailing, adaptor ligation, and PCR. Libraries were generated and sequenced on a HiSeq 4000 on a HiSeq 4000, (Illumina, San Diego, CA, USA) using a HiSeq 4000 PE Cluster Kit (PN PE-410-1001) with HiSeq 4000 SBS Kit (100 cycles, PN FC-410-1002) following manufacturer recommended protocols. Cell Ranger Single-Cell Software Suite 1.3 (10x Genomics, Pleasanton, CA, USA) was used to perform sample de-multiplexing, barcode processing, and single-cell gene counting (alignment, barcoding, and unique molecular identifier (UMI) count) at the University of Michigan Biomedical Core Facilities DNA Sequencing Core (52).

Bioinformatics Analysis of Single-cell RNA Sequencing Data

Analysis was performed as previously described (52). Briefly, a total of ~200 million reads were generated from the 10x Genomics sequencing analysis for each of the replicates. The sequencing data were first preprocessed using the 10x Genomics software Cell Ranger and aligned to mm10 genome (GEO accession: GSE144270). Quality control and analysis was performed as previously described (52). Downstream analysis steps were performed using Seurat pipeline (53) and included normalization, scaling, dimensionality reduction (principal component analysis [PCA] and t-distributed stochastic neighbor embedding [t-SNE]), unsupervised clustering, cluster consolidation via centroid rank correlation analysis, discovery of differentially expressed cluster specific markers, and marker enrichment. Presence of replicate batch effect was excluded by visual inspection of the contribution of each replicate to the PCA and t-SNE projections of the group. Provisional

clusters were assigned via unsupervised clustering (Seurat FindClusters, Louvain algorithm, $k = 30$, resolution = 0.4), resulting in 21 and 17 provisional clusters for the case and control group, respectively. Provisional clusters were aligned according to the rank correlation of their centroids, measured on the gene set derived from the intersection of the genes for each set. Final clusters were obtained by grouping similar clusters based on centroid rank correlation analysis. Clusters appear to be well distinguished in the PCA and t-SNE projections (52). Characteristic gene expression and cluster name abbreviation key is provided in **Supp. Table 2** and previously published data by Stepien, et al. (52)

Statistical Analysis

Statistical tests were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) software. Histological quantification of stained images were subjected to Student *t*-test or ANOVA with multiple comparisons post-test. Cross-sectional area and Feret diameter were represented as frequency of distribution graphed in percent frequency and compared using Komolgorov-Smirnov analysis. All data are presented as mean values with standard deviation (SD), and *P* values <0.05 were considered to be statistically significant.

Results

Ischemia reperfusion injury resulted in fibrosis and NET formation in the skeletal muscle

To recapitulate skeletal muscle fibrosis following IR injury, muscle injury and fibrosis were investigated in the injured tibialis anterior muscle and contralateral healthy control of C57BL/6 mice. Skeletal muscle injury was characterized by intense leukocyte infiltration at 3 DPI with lingering necrotic myofibers as indicated by H&E staining (**Supp. Fig. 1**). At 3 DPI, picrosirius red staining revealed loose, disorganized collagen deposition (**Supp. Fig. 1**) and by 7 DPI, strong collagen deposition was present between regenerating myofibers, a pattern consistent with fibrotic deposition (**Fig 1, Supp. Fig. 1**). By 7 DPI, necrotic tissue was replaced by regenerating skeletal muscle myofibers with prominent centralized nuclei, cytoplasmic basophilia, and oblong shape with smooth edges (**Fig 1, Supp. Fig. 1**). IR injury in the skeletal muscle was also characterized by a robust immune system response. To evaluate the formation of NETs in the skeletal muscle following IR injury, muscle sections at 1 and 3 DPI were immunofluorescently labeled with H3Cit, MPO, and Hoechst and NETs were identified. Robust NET formation was demonstrated in the skeletal muscle following IR injury (**Fig 1, Supp. Fig. 2**, indicated by white arrowheads). NETs were not present in uninjured skeletal muscle, as shown in **Supp. Fig. 2**.

Decrease of NETosis via PAD4 pathway did not alter muscle regeneration or fibrosis

Protein arginine deiminase 4 (PAD4) is required for NETosis that occurs through citrullination of histone H3 and H4 (48, 54-57). NETs formed in the muscle in the mouse model of IR demonstrate citrullination of histone H3 (**Supp. Fig. 2**), thus PAD4^{-/-} mice and the small molecule PAD inhibitor Cl-amidine were utilized to

investigate the role of NETs formed via the PAD4 pathway in fibrotic deposition from IR injury. C57BL/6 mice underwent IR injury and were administered Cl-amidine or vehicle treatment via intraperitoneal injection daily. TA muscles were harvested at 3 and 7 DPI, flash frozen, cryosectioned, and histologically processed to evaluate NET formation, myofiber regeneration, and muscle fibrosis. NET formation was quantified at 3 DPI. Daily administration of Cl-amidine led to a significant decrease in NET formation from 24.1 ± 11.7 to 3.9 ± 2.7 following treatment (NETs per 63x ROI, $n=3$ per group; $P<0.05$; **Fig 1A**). Mice treated with Cl-amidine presented with an increased frequency of myofibers with small CSA and Feret diameter ($P<0.0001$, both analyses), indicating that administration of Cl-amidine hindered muscle regeneration at 7 DPI compared to vehicle (**Fig. 1B**). Similarly, Cl-amidine did not have an effect on muscle fibrosis at 7 DPI compared to the vehicle ($17.5 \pm 0.41\%$; vs $50.6\% \pm 5.5$ vs $47.7\% \pm 11.1$ uninjured, Cl-amidine, vehicle, respectively; mean $\pm$ SD $P=0.8$, $n=3-6$ /group; ordinary one-way ANOVA plus Tukey's test). (**Fig. 1C**). As pharmacological intervention may not completely inhibit the PAD4 signaling, we next sought to investigate this phenomenon in a transgenic mouse lacking PAD4. To ensure a robust injury and closely mimic the polytrauma injuries that often accompany IR, in the complimentary transgenic studies IR injury was performed as described and 10 μ L of 3 mg/ml ctx was also injected into the injured tibialis anterior immediately following clamp release. Results in the PAD4^{-/-} mice were similar to that of the pharmacological inhibition. Muscle regeneration in the PAD4^{-/-} mouse was decreased compared to the wild type control, as evidenced by an increased relative frequency of myofibers with small Feret diameter ($P<0.0001$; **Fig. 1D**). Muscle fibrosis was also similar between the PAD4^{-/-} mice and wild type C57BL/6 control mice at 7 DPI ($35.0\% \pm 4.25$ vs $39.8\% \pm 6.7$; PAD4^{-/-}, C57BL/6 wild type control respectively, mean $\pm$ SD, $P=0.34$, unpaired t -test, **Fig. 1E**). Despite a decrease in NET formation, mice treated with Cl-amidine did not have a significant difference in myofiber regeneration, as assessed by myofiber CSA and Feret diameter. To identify if any treatments or genotypes provided protective effects on the myofibers, we compared the percent of myofibers possessing centralized nuclei (indicating regenerating myofibers) between groups and the percent of myofibers exhibiting nuclei on the periphery of the myofiber (indicating an uninjured/protected myofiber). We found similar composition between vehicle and Cl-amidine treatment (regenerating: $0.05\% \pm 0.08\%$ vs $99.7\% \pm 0.24$ vs $99.7\% \pm 0.14$; and uninjured/protected: 99.95 ± 0.082 vs $0.35\% \pm 0.24$ vs $0.28\% \pm 0.14\%$; uninjured, Cl-amidine, vehicle, respectively, $n=3-6$ /group, ANOVA followed by Tukey's test), as shown in **Fig 1F**. Similarly, both C57BL/6 and PAD4^{-/-} muscles were comprised of nearly 100% regenerating fibers without substantial peripherally located nuclei (**Fig 1G**). This data indicated that PAD4 inhibition both pharmacologically and genetically did not affect the initial injury to the TA muscle or provide protective benefits to the myofibers. All these findings taken together suggest that NET formation via the PAD4 mechanism was not essential in fibrotic deposition in the skeletal muscle following IR injury.

IR injury induced IL-10 and TNF α production

NETs and TLR7/8/9 have been linked to secondary self-NET propagation and have been shown to augment circulating levels of proinflammatory cytokines IL-10 and tumor necrosis factor- α (TNF α) (32, 58). In our rat model of hind limb tourniquet induced IR injury (59, 60), we show that IL-10 levels were significantly elevated at 6 and 24 hr post injury from 758 ± 244 pg/ml in uninjured animals to 1753 ± 121 pg/ml at 6 hr ($P < 0.0001$) and 1659 ± 213 pg/ml at 24 hr ($P < 0.0001$) (**Fig. 2A**). We found similar elevations in TNF α at 6 and 24 hr post injury from 2633 ± 1062 pg/ml to 7047 ± 1202 pg/ml ($P < 0.0001$) and 6717 ± 1183 pg/ml ($P < 0.0001$), respectively (**Fig. 2A**), suggesting that IR injury mimicked other NET-related disease cytokine release, and similar TLR7/8/9 mediated pathways may have also been involved. To understand the cell populations that were involved in TLR7/8/9, IL-10 and TNF α release, we conducted a transcriptome analysis of the cell types at 3 DPI involved in skeletal muscle regeneration using single-cell sequencing (scRNA-seq) in a mouse model of polytrauma (IR+ctx), which has been shown to produce a robust inflammatory reaction that results in ECM deposition, myonecrosis, and ultimately fibrosis (52). Unsupervised clustering identified 11 distinct cellular clusters in uninjured muscle and 8 in IR+ctx (**Fig 2B-C, Supp. Table 2**). Uninjured muscle was largely composed of endothelial cells/vascular progenitor cells, FAPs, satellite cells, and *scleraxis* expressing mesenchymal stem cells. Injured muscle also contained an FAP population, as well as a greater predominance of immune cells including macrophages, dendritic cells, and granulocytes. Of note, the uninjured muscle was found to contain a *Pax7* positive satellite cell population, indicating quiescent satellite cells, while the injured muscle contained a myoblast population, defined by *Myf5* and *Myod1*. *Il10* was expressed nearly exclusively by the macrophage population (**Fig 2D-E**) and *Tnfa* at highest levels in the granulocyte population (**Fig 2F-G**). These results indicate that traumatic injury induced *Il10* and *Tnfa* gene expression at the same time point as robust NET formation and that NETs present in the tissue following injury may stimulate TLR7/8/9 pathways, leading to increased IL10 and TNF α levels observed here.

Treatment with HCQ suppressed IL-10 and TNF α production

We next sought to modulate TLR signaling pathway to mitigate injury caused by IR. HCQ has been shown effective in NET and RNA mediated diseases and viral infections, and is known to lower IL-10 and TNF α levels in lupus patients as well as decrease disease severity (61). Ischemia reperfusion injured C57BL/6 mice treated systemically with 60 mg/kg HCQ daily had a trend towards lower IL-10 and significantly lower TNF α ($P < 0.05$) plasma levels (**Fig. 2H-I**) at 3 DPI, which correlates with the detected robust NET formation and presence of *Il10* and *TNfa* expressing immune cells (**Fig 1A, Fig. 3C-G**). Interestingly, no significant differences in other measured pro-inflammatory cytokine were detected (**Supp. Table 3**).

HCQ treatment decreased NET formation

Given that HCQ acts on TLR7/8/9, we evaluated NET formation at 3 DPI in C57BL/6 mice treated with vehicle or HCQ. Mice treated with HCQ showed substantial trend towards decreased NET formation, (NETs per 63x

ROI: HCQ: 4.5 ± 1.6 , vehicle: 7.4 ± 3.2 ; $n=6/\text{group}$; $P=0.08$, unpaired *t*-test) (**Fig. 3A**). This potential decrease in NET formation in the HCQ treated mice is unlikely to be due to differences in initial neutrophil recruitment, as treatment with HCQ did not show a difference in neutrophil recruitment to the injured muscle at 3 DPI, as assessed by immunofluorescent staining for Ly6G (**Supp. Fig 3**). This trend of decreased NET formation following administration of TLR7/8/9 inhibitor further indicated that NET formation is likely partially, but not completely, mediated by TLR signaling following traumatic injury.

Treatment with HCQ attenuated fibrosis and improved myofiber regeneration following IR injury

Given the trend towards decreased NET formation observed following HCQ administration, we next sought to investigate the role of HCQ in muscle regeneration and fibrosis after IR injury. C57BL/6 mice underwent IR injury and were treated with vehicle or HCQ. Mice treated with HCQ presented with significantly decreased muscle fibrosis at 7 DPI than those treated with vehicle ($18.5\% \pm 2.3$ vs $30.4\% \pm 5.4$ vs $40.5\% \pm 4.0$; uninjured, HCQ, vehicle, respectively; $n=6/\text{group}$, $P<0.01$, ordinary one-way ANOVA plus Tukey's test) (**Fig. 3B**). Additionally, treatment with HCQ improved muscle regeneration. HCQ decreased the frequency of myofibers with small CSA and small Feret diameters (both analyses $P<0.0001$) at 5 DPI (**Fig 3C**). In order to determine if the differences seen in fibrosis and regeneration in the HCQ treated animals was a result of less severe initial injury, we again compared the percentage of regenerating (centralized nuclei) and uninjured/protected (peripheral nuclei) myofibers in each treatment group to assess if HCQ provided protective effects on the myofibers. The percentage of regenerating myofibers was not significantly different between vehicle and HCQ treated animals (0% vs $98.37\% \pm 2.08$ vs $98.78\% \pm 0.49$; uninjured, HCQ, vehicle, respectively, $n=6/\text{group}$, ANOVA followed by Tukey's test), as shown in **Fig 3D**. HCQ did not prevent initial injury to the myofibers either, as the composition of peripherally located myofibers was not significantly different between vehicle and HCQ treated animals (100% vs $1.63\% \pm 2.08$ vs $1.21\% \pm 0.49$; uninjured, HCQ, vehicle, respectively, $n=6/\text{group}$, ANOVA followed by Tukey's test) (**Fig 3D**). Taken together, this data indicated that HCQ significantly decreased fibrosis and increased regeneration following muscle IR injury. Furthermore, our data indicated that muscle fibrosis was at least in part independent of NET formation in this injury, as the increase in regeneration and reduction in fibrosis was accompanied by a non-significant decrease in NET formation.

HCQ Treatment Did Not Induce Mitochondrial Damage

Antimalarial myopathy and mitochondrial abnormalities have been reported after chronic chloroquine regimens (62). In order to assess the presence of a mitochondrial myopathy in the injured TA following HCQ treatment, COX/SDH double staining was performed. The COX/SDH stain was used to detect fibers that lacked COX activity staining, but in which positive SDH staining was present (COX-/SDH+), which is indicative of mitochondrial myopathies (63, 64), or fibers that are both COX and SDH positive. No myofibers were found to lack COX staining and a significant difference was not found between the proportion of COX+/SDH+

myofibers of muscles treated with HCQ compared to vehicle treated, as shown in **Supp. Fig. 4**, which indicated that 7 days of daily HCQ treatment did not induce significant mitochondrial dysfunction in the injured muscles.

HCQ Treatment Inhibited FAP Proliferation

FAP cells proliferate following injury and differentiate into myofibroblasts, which are responsible for ECM deposition and fibrotic deposition (47). Having shown the HCQ decreased ECM deposition and fibrosis following IR injury, we hypothesized that HCQ may be acting on FAPs. Indeed, staining for FAPs (identified by PDGFR α expression) and the proliferation marker Ki67 at 5 DPI revealed that HCQ treatment significantly decreased the proliferation in FAP cells, as shown by a decreased number of double-positive PDGFR α -Ki67 cells in the HCQ treated muscles compared to vehicle treated (0.1 ± 0.3 vs 2.6 ± 1.6 vs 8.5 ± 4.7 ; uninjured, HCQ, vehicle, respectively; $n=4-6/\text{group}$, $P<0.0001$, ordinary one-way ANOVA plus Tukey's test) (**Fig 4A-B**). Furthermore, we used linear regression analysis to correlate total PDGFR α staining with total Ki67 staining and demonstrated that vehicle treated animals showed a significant linear correlation between PDGFR α and Ki67 stains ($P=0.04$, **Fig 4E**), which suggested that FAPs were the proliferating population. By contrast, there was poor concordance of FAP/proliferation correlation plots of the HCQ treated animals ($P=0.17$ **Fig 4D**), which suggested that, while Ki67 staining was present, it did not correlate to increased PDGFR α expression. Uninjured TA muscles presented with few FAPs and decreased total staining of both PDGFR α and Ki67 (**Fig 4A-C**). Following injury, FAPs may also differentiate to adipocytes (47). Oil Red O staining, which identifies lipid deposition, in the vehicle and HCQ treated TAs at 5 DPI identified very little lipid accumulation, indicating that FAPs were not driven towards adipogenesis following IR injury, nor did HCQ lead to adipocyte formation (**Supp. Fig. 5**). Taken together, this indicated that treatment with HCQ decreased proliferation in the FAP population at 5 DPI, which corresponded to a decrease in fibrosis at 7 DPI.

Treatment with HCQ inhibited ERK1/2 phosphorylation after IR injury

The role of ERK signaling following IR injury is not yet completely elucidated, but most studies to date show decreased ERK signaling and/or decreased ERK1/2 phosphorylation in the injured tissue to be beneficial for tissue survival and regeneration (38-43) and ERK signaling has also been implicated in fibrotic diseases (36, 37, 65-67). In order to investigate the role of ERK signaling in IR injury, we analyzed the IR+ctx single-cell data set to characterize *Erk1* and *Erk2* gene expression in uninjured and injured skeletal muscle without treatment interventions. Given the central role in proliferation, transcription regulation, and differentiation, both genes were widely expressed both in the uninjured and injured muscle at 3 DPI (**Fig. 5A-J**). Interestingly, *Erk1* and *Erk2* were differentially expressed in the FAP cell population (characteristic genes provided in **Supp. Table 2 and Supp. Fig 6**) after injury (**Fig. 5C, H**). FAPs expand in response to injury and contribute to fibrotic deposition by myofibroblasts, and an increase in fibrotic gene expression (including *Col3a1*, *Colla2*, *Snail*,

Ctgf) at this time point has previously been shown in this population (52, 68, 69). A full analysis of *Erk1* and *Erk2* expression levels in each cluster of uninjured and injured muscle is included in **Supp. Fig. 7**. Of note, little *Erk1* or *Erk2* expression was present in the granulocyte population of the IR+ctx muscle, the population from which NETs would arise (**Supp. Fig. 7**). Furthermore, low levels *Erk1* and *Erk2* expression was present in the satellite cell population of the uninjured satellite cell population (*Pax7*⁺, *Chodl*⁺, *Sdc4*⁺), however expression of *Erk1* and *Erk2* was present in the myoblast population (*Myod1*⁺, *Myf5*⁺) of the IR+ctx injured muscle at 3 DPI (**Supp. Fig. 7**). We next investigated *Erk1* and *Erk2* expression at 7 DPI in muscles treated with vehicle or HCQ. At 7 DPI, at which point a decrease in fibrotic deposition was seen in HCQ-treated mice, *Erk1* and *Erk2* gene expression was similar in whole muscle tissue of vehicle or HCQ treated mice (**Fig. 5K-L**), which indicated that treatment with HCQ does not significantly change genetic expression of *Erk1* or *Erk2*. Additional genes of interest are presented in **Supp. Table 4**. Similarly, whole tissue protein analysis by western blot showed similar levels of ERK1/2 protein in tissues treated with vehicle or HCQ at 7 DPI (**Fig. 5M**). However, animals treated with HCQ showed a significantly lower ratio of pERK1/2 to ERK1/2 than the vehicle treated mice (vehicle: 0.98 ± 0.07 ; HCQ: 0.61 ± 0.21 , $n=3/\text{group}$, $P<0.05$, unpaired *t*-test), which indicated that treatment with HCQ decreased ERK1/2 phosphorylation in the injured muscle tissue (**Fig. 5M-N**). Concurrently at 7 DPI, mice treated with HCQ also presented with decreased muscle fibrosis, suggesting that inhibition of ERK1/2 phosphorylation contributed to decreased muscle fibrosis. At 7 DPI, *Erk1* and *Erk2* gene expression were similar in bulk muscle treated with vehicle or HCQ, however, HCQ inhibited ERK1/2 phosphorylation in the muscle tissue, which corresponded to a decrease in muscle fibrosis.

Discussion

Following a period of ischemia and subsequent reperfusion, devastating fibrosis is often formed in skeletal muscle (3). Here, we recapitulated this injury in a mouse model of hindlimb IR injury. We showed that IR injury resulted in extensive immune infiltration post injury and led to fibrotic deposition by 7 DPI. As part of the initial immune response, we identified robust NET formation in the skeletal muscle. We found that modulation of TLR7/8/9 signaling by administration of HCQ following injury lowered circulating TNF α levels, showed a trend to lower IL-10 levels, significantly improved muscle regeneration, and attenuated muscle fibrosis. This effect was at least in part independent of NET formation via TLR7/8/9 signaling as NET formation in the HCQ treated mice was not significantly decreased. Upon further investigation, we identified that HCQ decreased proliferation in FAP cells and modulated ERK1/2 phosphorylation, a pathway known to be involved in the fibrotic response to IR injury. Administration of HCQ may be an effective treatment to mitigate muscle damage and fibrosis following IR injury.

Following IR injury, we identified extensive neutrophil infiltration into the injured muscle and robust NET formation in the injured skeletal muscle. NETs are present in other models of trauma, and are known to

exacerbate the injury in many cases (1, 4, 5, 70). For example, following a 30% total body surface area burn in combination with an Achilles tendon tenotomy, mice exhibit robust NET formation at the tenotomy site at three days post injury (17). A mouse model of antibody-mediated transfusion-related acute lung injury also shows NETs in the alveoli of injured mice, and following an experimental model of murine deep vein thrombosis in the inferior vena cava, NETs are present in the thrombus and contribute to thrombosis (18, 19). Therefore, in the current study, we investigated if NET formation following IR injury may also negatively affect tissue regeneration and contribute to fibrosis. Classically, NETs are formed through suicidal NETosis and citrullination of histone H3, a pathway dependent on PAD4. Thus, we inhibited PAD4 pharmacologically as well as deleted PAD4 genetically to investigate the role of suicidal NETosis in muscle fibrosis. While NETs were decreased after Cl-amidine treatment, interestingly, little difference was seen in fibrotic deposition after pharmacological or genetic deletion of PAD4. These results suggested that NET formation via the PAD4 mechanism did not play an essential role in fibrotic deposition in the skeletal muscle following IR injury. However, NETs have also been shown to form through a TLR 7/8/9 mediated pathway that is independent of PAD4 via secondary self-propagation (17, 24). That is, nucleic acid material present in the tissue stimulates the TLR7/8/9 receptors of immune cells, which triggers additional inflammation and a second wave of neutrophils and NETs. We have shown that NETs were abundant in the injured tissue, which may go on to contribute to secondary NETosis. Additionally, while not explicitly investigated here, it is reasonable to hypothesize that free nucleic material was present in the tissue due to mechanical trauma (21, 71). Thus, we investigated TLR7/8/9 signaling further in the context of muscle fibrosis and NET formation.

NETs are perhaps best characterized in autoimmune diseases such as lupus and rheumatoid arthritis, and we saw several similarities between the IR injury and these diseases, despite the vastly different underlying pathologies. First, we showed that the immune response to IR injury led to elevated serum levels of IL-10 and TNF α , which are associated with TLR7/8/9 stimulation and NETs. For example, NETs formed upon activation of neutrophils with *Mycobacterium tuberculosis*, which acts through TLR7/8/9 activation, stimulate macrophages and monocytes to produce IL-10 and TNF α (72). High serum levels of IL-10 and TNF α are also implicated with rheumatoid arthritis and lupus, both diseases characterized by excessive NET production (11-13, 32). In fact, increased levels of IL-10 in lupus patients has been correlated to increased disease severity (73). We found that in our mouse model of IR injury, high serum levels of IL-10 and TNF α corresponded to the time point at which NETs are present in the injured tissue (3 DPI). Using scRNA-seq in the IR+ctx model, we identified that *Il10* was expressed by the macrophage population, and *Tnf α* largely by the granulocyte population, indicating that the infiltrating immune cells were responsible for increased circulating IL-10 and TNF α levels. Interestingly, pulmonary fibrosis can be seen in lupus, and NETs are also abundant in the airway fluids of cystic fibrosis patients, further suggesting a link between NETs and fibrosis (74). Therefore, we chose to investigate HCQ, a small molecule TLR7/8/9 inhibitor that is prescribed for lupus and rheumatoid arthritis

patients and has been shown to decrease TNF α serum levels in NET-associated diseases (75, 76). Indeed, delivery of the TLR7/8/9 inhibitor HCQ to mice following IR injury lowered TNF α levels as well as IL-10. Thus, we hypothesized that administration of HCQ following IR injury may also affect NET formation and muscle injury.

We found that HCQ significantly improved muscle regeneration and decreased muscle fibrosis following IR injury, however, HCQ treatment resulted in a non-significant decrease in NET formation. HCQ is also known to inhibit TLR7/8 signaling as well as have effects on autophagy and metabolic activity, both of which may contribute to the pro-regenerative and anti-fibrotic effect seen here (77, 78). We have also shown that treatment with HCQ decreases proliferation in the FAP cell population, which differentiate to myofibroblasts and are ultimately responsible for fibrotic deposition (47). While further studies are needed to elucidate the cell specific actions of HCQ, we hypothesize that the decrease in FAP proliferation after HCQ treatment is at least partially responsible for the concordant decrease fibrotic deposition.

A growing body of research also ties *Mapk* signaling and ERK1/2 activation to fibrosis, and inhibition of ERK1/2 is shown to attenuate fibrosis (36, 37, 65-67). Here, we showed that differential *Erk1* and *Erk2* signaling in the FAP population of IR+ctx injured skeletal muscle correlated to increased fibrotic gene expression in the FAP population, which suggested a correlation between *Mapk* signaling and fibrosis (52). Thus, we investigated the role of *Erk1* and *Erk2* gene expression and ERK1/2 protein levels and phosphorylation in IR injury. We found that administration of HCQ decreased ERK1/2 phosphorylation at 7 DPI, which corresponded to decreased fibrosis. *Erk1* and *Erk2* expression in the muscle was similar in muscle treated with HCQ or vehicle, as were the levels of ERK1/2 protein, which indicated that the activation and subsequent phosphorylation of ERK1/2 were likely the important step in the ERK signaling process directing fibrosis. Further investigation into the role of ERK1/2 activation and related pathways will be an important next step to characterize the role of HCQ treatment in muscle fibrosis.

We have identified that treatment with FDA-approved HCQ mitigated muscle fibrosis and improved myofiber regeneration following IR injury, suggesting HCQ may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury. Although chloroquine-induced myopathy has been reported, this side effect is rare and reversible once the dosing regimen is stopped (62, 79, 80). We found that daily dosing of 60 mg/kg HCQ for 7 days did not lead to mitochondrial dysfunction in the injured muscle, as assessed by COX/SDH double staining. Overall, HCQ decreased IL-10, TNF α , FAP proliferation, and ERK1/2 phosphorylation, which contributed to the pro-regenerative and anti-fibrotic effects observed. Administration of HCQ resulted in a modest decrease of NETs in the skeletal muscle, which suggested that the muscle fibrosis following IR injury was at least partially independent of NET formation. Similarly, mice with PAD4 pharmacologically inhibited or genetically deleted and therefore lacking NETs formed via citrullination of

histone H3 did not present with differences in muscle fibrosis, which suggested fibrosis is independent of NET formation via PAD4. These insights into the role of NETs in fibrotic injuries and mechanisms of HCQ may offer important therapies in addition to HCQ and contribute to understanding the role of NETs in traumatic and fibrotic injuries. Further investigation will focus on the mechanism of action of HCQ in muscle fibrosis.

References

1. Gillani, S., Cao, J., Suzuki, T., and Hak, D.J., (2012) The effect of ischemia reperfusion injury on skeletal muscle. *Injury*. **43**(6): 670-5
2. de Groot, H. and Rauen, U., (2007) Ischemia-reperfusion injury: processes in pathogenetic networks: a review. *Transplant Proc.* **39**(2): 481-4
3. David Cholak, E.L., MD, Jeffrey Lisiecki, MD, Shailesh Agarwal, MD, Shawn Loder, MD, Kavitha Ranganathan, MD, Ammar T. Qureshi, PhD, and Thomas A. Davis, P., Benjamin Levi, MD, (2016) Traumatic muscle fibrosis: From pathway to prevention. *J Trauma Acute Care Surg.* **82**(1): 174-184
4. Kalogeris, T., Baines, C.P., Krenz, M., and Korthuis, R.J., (2012) Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol.* **298**: 229-317
5. Oklu, R., Albadawi, H., Jones, J.E., Yoo, H.J., and Watkins, M.T., (2013) Reduced hind limb ischemia-reperfusion injury in Toll-like receptor-4 mutant mice is associated with decreased neutrophil extracellular traps. *J Vasc Surg.* **58**(6): 1627-36
6. Albadawi, H., Oklu, R., Raacke Malley, R.E., O'Keefe, R.M., Uong, T.P., Cormier, N.R., and Watkins, M.T., (2016) Effect of DNase I treatment and neutrophil depletion on acute limb ischemia-reperfusion injury in mice. *J Vasc Surg.* **64**(2): 484-493
7. Patel, R., Albadawi, H., Steudel, W., Hashmi, F.F., Kang, J., Yoo, H.J., and Watkins, M.T., (2012) Inhalation of carbon monoxide reduces skeletal muscle injury after hind limb ischemia-reperfusion injury in mice. *Am J Surg.* **203**(4): 488-95
8. Honda, M., Takeichi, T., Asonuma, K., Tanaka, K., Kusunoki, M., and Inomata, Y., (2013) Intravital imaging of neutrophil recruitment in hepatic ischemia-reperfusion injury in mice. *Transplantation.* **95**(4): 551-8
9. de Oliveira, T.H.C., Marques, P.E., Poosti, F., Ruytinx, P., Amaral, F.A., Brandolini, L., Allegretti, M., Proost, P., and Teixeira, M.M., (2017) Intravital Microscopic Evaluation of the Effects of a CXCR2 Antagonist in a Model of Liver Ischemia Reperfusion Injury in Mice. *Front Immunol.* **8**: 1917
10. Volker Brinkmann, U.R., Christian Goosmann, Beatrix Fauler, Yvonne Uhlemann, David S. Weiss, Yvette Weinrauch, Arturo Zychlinsky, (2004) Neutrophil Extracellular Traps Kill Bacteria. *Science.* **303**
11. Knight, J.S., Subramanian, V., O'Dell, A.A., Yalavarthi, S., Zhao, W., Smith, C.K., Hodgins, J.B., Thompson, P.R., and Kaplan, M.J., (2015) Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Ann Rheum Dis.* **74**(12): 2199-206
12. Knight, J.S., Zhao, W., Luo, W., Subramanian, V., O'Dell, A.A., Yalavarthi, S., Hodgins, J.B., Eitzman, D.T., Thompson, P.R., and Kaplan, M.J., (2013) Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest.* **123**(7): 2981-93
13. Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., Meller, S., Chamilos, G., Sebasigari, R., Riccieri, V., Bassett, R., Amuro, H., Fukuhara, S., Ito, T., Liu, Y.J., and Gilliet, M.,

- (2011) Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med.* **3**(73): 73ra19
14. Khandpur, R., Carmona-Rivera, C., Vivekanandan-Giri, A., Gizinski, A., Yalavarthi, S., Knight, J.S., Friday, S., Li, S., Patel, R.M., Subramanian, V., Thompson, P., Chen, P., Fox, D.A., Pennathur, S., and Kaplan, M.J., (2013) NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med.* **5**(178): 178ra40
 15. Willis, V.C., Banda, N.K., Cordova, K.N., Chandra, P.E., Robinson, W.H., Cooper, D.C., Lugo, D., Mehta, G., Taylor, S., Tak, P.P., Prinjha, R.K., Lewis, H.D., and Holers, V.M., (2017) Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. *Clin Exp Immunol.* **188**(2): 263-274
 16. Wong, S.L. and Wagner, D.D., (2018) Peptidylarginine deiminase 4: a nuclear button triggering neutrophil extracellular traps in inflammatory diseases and aging. *FASEB J.* **32**: 6358-6370
 17. Agarwal, S., Loder, S.J., Cholok, D., Li, J., Bian, G., Yalavarthi, S., Li, S., Carson, W.F., Hwang, C., Marini, S., Pagani, C., Edwards, N., Delano, M.J., Standiford, T.J., Knight, J.S., Kunkel, S.L., Mishina, Y., Ward, P.A., and Levi, B., (2019) Disruption of Neutrophil Extracellular Traps (NETs) Links Mechanical Strain to Post-traumatic Inflammation. *Front Immunol.* **10**: 2148
 18. Brill, A., Fuchs, T.A., Savchenko, A.S., Thomas, G.M., Martinod, K., De Meyer, S.F., Bhandari, A.A., and Wagner, D.D., (2012) Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost.* **10**(1): 136-44
 19. Caudrillier, A., Kessenbrock, K., Gilliss, B.M., Nguyen, J.X., Marques, M.B., Monestier, M., Toy, P., Werb, Z., and Looney, M.R., (2012) Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* **122**(7): 2661-71
 20. Itagaki, K., Kaczmarek, E., Lee, Y.T., Tang, I.T., Isal, B., Adibnia, Y., Sandler, N., Grimm, M.J., Segal, B.H., Otterbein, L.E., and Hauser, C.J., (2015) Mitochondrial DNA released by trauma induces neutrophil extracellular traps. *PLoS One.* **10**(3): e0120549
 21. McIlroy, D.J., Jarnicki, A.G., Au, G.G., Lott, N., Smith, D.W., Hansbro, P.M., and Balogh, Z.J., (2014) Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery. *J Crit Care.* **29**(6): 1133 e1-5
 22. Allen, C., Thornton, P., Denes, A., McColl, B.W., Pierozynski, A., Monestier, M., Pinteaux, E., Rothwell, N.J., and Allan, S.M., (2012) Neutrophil cerebrovascular transmigration triggers rapid neurotoxicity through release of proteases associated with decondensed DNA. *J Immunol.* **189**(1): 381-92
 23. Kenny, E.F., Herzig, A., Kruger, R., Muth, A., Mondal, S., Thompson, P.R., Brinkmann, V., Bernuth, H.V., and Zychlinsky, A., (2017) Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife.* **6**
 24. Herster, F., Bittner, Z., Archer, N.K., Dickhofer, S., Eisel, D., Eigenbrod, T., Knorpp, T., Schneiderhan-Marra, N., Löffler, M.W., Kalbacher, H., Vierbuchen, T., Heine, H., Miller, L.S., Hartl, D., Freund, L., Schakel, K., Heister, M., Ghoreschi, K., and Weber, A.N.R., (2020) Neutrophil extracellular trap-associated RNA and LL37 enable self-amplifying inflammation in psoriasis. *Nat Commun.* **11**(1): 105
 25. Barton, G.M., Kagan, J.C., and Medzhitov, R., (2006) Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol.* **7**(1): 49-56
 26. Carmelo Carmona-Rivera, P.M.C., Erica Moore, Nithya Lingampalli, Hannes Uchtenhagen, E.J., Yudong Liu, Kevin L. Bicker, Heidi Wahamaa, Victoria Hoffmann, A.I.C., Paul R. Thompson, Jane H. Buckner, and William H. Robinson, D.A.F., Mariana J. Kaplan, (2017) Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci. Immunol.* **2**
 27. Lindau, D., Mussard, J., Rabsteyn, A., Ribon, M., Kotter, I., Igney, A., Adema, G.J., Boissier, M.C., Rammensee, H.G., and Decker, P., (2014) TLR9 independent interferon alpha production by neutrophils on NETosis in response to circulating chromatin, a key lupus autoantigen. *Ann Rheum Dis.* **73**(12): 2199-207
 28. Liu, L., Mao, Y., Xu, B., Zhang, X., Fang, C., Ma, Y., Men, K., Qi, X., Yi, T., Wei, Y., and Wei, X., (2019) Induction of neutrophil extracellular traps during tissue injury: Involvement of STING and Toll-like receptor 9 pathways. *Cell Prolif.* e12579

29. Mallavia, B., Liu, F., Lefrancais, E., Cleary, S.J., Kwaan, N., Tian, J.J., Magnen, M., Sayah, D.M., Soong, A., Chen, J., Sagggar, R., Shino, M.Y., Ross, D.J., Derhovanessian, A., Lynch Iii, J.P., Ardehali, A., Weigt, S.S., Belperio, J.A., Hays, S.R., Golden, J.A., Leard, L.E., Shah, R.J., Kleinhenz, M.E., Venado, A., Kukreja, J., Singer, J.P., and Looney, M.R., (2019) Mitochondrial DNA Stimulates TLR9-Dependent NET Formation in Primary Graft Dysfunction. *Am J Respir Cell Mol Biol.* **62**(3): 364-372
30. Nie, M., Yang, L., Bi, X., Wang, Y., Sun, P., Yang, H., Liu, P., Li, Z., Xia, Y., and Jiang, W., (2019) Neutrophil Extracellular Traps Induced by IL8 Promote Diffuse Large B-cell Lymphoma Progression via the TLR9 Signaling. *Clin Cancer Res.* **25**(6): 1867-1879
31. Chowdhury, C.S., Giaglis, S., Walker, U.A., Buser, A., Hahn, S., and Hasler, P., (2014) Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther.* **16**(R122): 1-14
32. Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., Punaro, M., Baisch, J., Guiducci, C., Coffman, R.L., Barrat, F.J., Banchereau, J., and Pascual, V., (2011) Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med.* **3**(73): 73ra20
33. Hakkim, A., Furnrohr, B.G., Amann, K., Laube, B., Abed, U.A., Brinkmann, V., Herrmann, M., Voll, R.E., and Zychlinsky, A., (2010) Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A.* **107**(21): 9813-8
34. Leffler, J., Ciacma, K., Gullstrand, B., Bengtsson, A.A., Martin, M., and Blom, A.M., (2015) A subset of patients with systemic lupus erythematosus fails to degrade DNA from multiple clinically relevant sources. *Arthritis Res Ther.* **17**: 205
35. Wang, L.F., Lin, Y.S., Huang, N.C., Yu, C.Y., Tsai, W.L., Chen, J.J., Kubota, T., Matsuoka, M., Chen, S.R., Yang, C.S., Lu, R.W., Lin, Y.L., and Chang, T.H., (2015) Hydroxychloroquine-inhibited dengue virus is associated with host defense machinery. *J Interferon Cytokine Res.* **35**(3): 143-56
36. Zhong, W., Shen, W.F., Ning, B.F., Hu, P.F., Lin, Y., Yue, H.Y., Yin, C., Hou, J.L., Chen, Y.X., Zhang, J.P., Zhang, X., and Xie, W.F., (2009) Inhibition of extracellular signal-regulated kinase 1 by adenovirus mediated small interfering RNA attenuates hepatic fibrosis in rats. *Hepatology.* **50**(5): 1524-36
37. Andrikopoulos, P., Kieswich, J., Pacheco, S., Nadarajah, L., Harwood, S.M., O'Riordan, C.E., Thiemermann, C., and Yaqoob, M.M., (2019) The MEK Inhibitor Trametinib Ameliorates Kidney Fibrosis by Suppressing ERK1/2 and mTORC1 Signaling. *J Am Soc Nephrol.* **30**(1): 33-49
38. Collier, J.B., Whitaker, R.M., Eblen, S.T., and Schnellmann, R.G., (2016) Rapid Renal Regulation of Peroxisome Proliferator-activated Receptor gamma Coactivator-1alpha by Extracellular Signal-Regulated Kinase 1/2 in Physiological and Pathological Conditions. *J Biol Chem.* **291**(52): 26850-26859
39. Okada, M., Yamane, M., Yamamoto, S., Otani, S., Miyoshi, K., Sugimoto, S., Matsukawa, A., Toyooka, S., Oto, T., and Miyoshi, S., (2018) SPRED2 deficiency may lead to lung ischemia-reperfusion injury via ERK1/2 signaling pathway activation. *Surg Today.* **48**(12): 1089-1095
40. Zhang, J., Xia, J., Zhang, Y., Xiao, F., Wang, J., Gao, H., Liu, Y., Rong, S., Yao, Y., Xu, G., and Li, J., (2016) HMGB1-TLR4 signaling participates in renal ischemia reperfusion injury and could be attenuated by dexamethasone-mediated inhibition of the ERK/NF-kB pathway. *Am J Transl Res.* **8**(10): 4054-4067
41. Zhou, Q.L., Teng, F., Zhang, Y.S., Sun, Q., Cao, Y.X., and Meng, G.W., (2018) FPR1 gene silencing suppresses cardiomyocyte apoptosis and ventricular remodeling in rats with ischemia/reperfusion injury through the inhibition of MAPK signaling pathway. *Exp Cell Res.* **370**(2): 506-518
42. Zhu, B.L., Xie, C.L., Hu, N.N., Zhu, X.B., and Liu, C.F., (2018) Inhibiting of GRASP65 Phosphorylation by DL-3-N-Butylphthalide Protects against Cerebral Ischemia-Reperfusion Injury via ERK Signaling. *Behav Neurol.* **2018**: 5701719
43. Xu, S., Niu, P., Chen, K., Xia, Y., Yu, Q., Liu, N., Li, J., Li, S., Wu, L., Feng, J., Wang, W., Lu, X., Liu, T., Wang, F., Dai, W., Fan, X., Mo, W., Xu, L., and Guo, C., (2017) The liver protection of propylene glycol alginate sodium sulfate preconditioning against ischemia reperfusion injury: focusing MAPK pathway activity. *Sci Rep.* **7**(1): 15175

44. Cai, Y., Wang, W., Qiu, Y., Yu, M., Yin, J., Yang, H., and Mei, J., (2018) KGF inhibits hypoxia-induced intestinal epithelial cell apoptosis by upregulating AKT/ERK pathway-dependent E-cadherin expression. *Biomed Pharmacother.* **105**: 1318-1324
45. Chen, Y., Ba, L., Huang, W., Liu, Y., Pan, H., Mingyao, E., Shi, P., Wang, Y., Li, S., Qi, H., Sun, H., and Cao, Y., (2017) Role of carvedilol in cardioprotection against myocardial ischemia/reperfusion injury in rats through activation of MAPK/ERK and Akt/eNOS signaling pathways. *Eur J Pharmacol.* **796**: 90-100
46. Choi, D.E., Jeong, J.Y., Choi, H., Chang, Y.K., Ahn, M.S., Ham, Y.R., Na, K.R., and Lee, K.W., (2017) ERK phosphorylation plays an important role in the protection afforded by hypothermia against renal ischemia-reperfusion injury. *Surgery.* **161**(2): 444-452
47. Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., Yoneda, T., Segawa, M., Yamaguchi, M., Ogawa, R., Matev, M.M., Miyagoe-Suzuki, Y., Takeda, S., Tsujikawa, K., Tsuchida, K., Yamamoto, H., and Fukada, S., (2011) Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J Cell Sci.* **124**(Pt 21): 3654-64
48. Li, P., Li, M., Lindberg, M.R., Kennett, M.J., Xiong, N., and Wang, Y., (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med.* **207**(9): 1853-62
49. Huang, L. and Wang, M.J., (1995) Image Thresholding By Minimizing The Measures of Fuzziness. *Pattern Recognit.* **28**(1): 41-51
50. Wen, Y., Murach, K.A., Vechetti, I.J., Jr., Fry, C.S., Vickery, C., Peterson, C.A., McCarthy, J.J., and Campbell, K.S., (2018) MyoVision: software for automated high-content analysis of skeletal muscle immunohistochemistry. *J Appl Physiol (1985).* **124**(1): 40-51
51. Ross, J.M., (2011) Visualization of mitochondrial respiratory function using cytochrome c oxidase/succinate dehydrogenase (COX/SDH) double-labeling histochemistry. *J Vis Exp*,(57): e3266
52. Stepien, D.M., Hwang, C., Marini, S., Pagani, C.A., Sorkin, M., Visser, N.D., Huber, A.K., Edwards, N.J., Loder, S.J., Vasquez, K., Aguilar, C.A., Kumar, R., Mascharak, S., Longaker, M.T., Li, J., and Levi, B., (2020) Tuning Macrophage Phenotype to Mitigate Skeletal Muscle Fibrosis. *J Immunol.* **204**(8): 2203-2215
53. Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R., (2018) Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol.* **36**(5): 411-420
54. Kusunoki, Y., Nakazawa, D., Shida, H., Hattanda, F., Miyoshi, A., Masuda, S., Nishio, S., Tomaru, U., Atsumi, T., and Ishizu, A., (2016) Peptidylarginine Deiminase Inhibitor Suppresses Neutrophil Extracellular Trap Formation and MPO-ANCA Production. *Front Immunol.* **7**: 227
55. Lewis, H.D., Liddle, J., Coote, J.E., Atkinson, S.J., Barker, M.D., Bax, B.D., Bicker, K.L., Bingham, R.P., Campbell, M., Chen, Y.H., Chung, C.W., Craggs, P.D., Davis, R.P., Eberhard, D., Joberty, G., Lind, K.E., Locke, K., Maller, C., Martinod, K., Patten, C., Polyakova, O., Rise, C.E., Rudiger, M., Sheppard, R.J., Slade, D.J., Thomas, P., Thorpe, J., Yao, G., Drewes, G., Wagner, D.D., Thompson, P.R., Prinjha, R.K., and Wilson, D.M., (2015) Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol.* **11**(3): 189-91
56. Sorensen, O.E. and Borregaard, N., (2016) Neutrophil extracellular traps - the dark side of neutrophils. *J Clin Invest.* **126**(5): 1612-1620
57. Yanming Wang, J.W., Joyce Sayegh, Young-Ho Lee, Julie R. Perlin, Lauriebeth Leonelli, Lakshmi S. Sonbuchner, Charles H. McDonald, Richard G. Cook, Yali Dou, Robert G. Roeder, Steven Clarke, Michael R. Stallcup, C. David Allis, Scott A. Coonrod, (2004) Human PAD4 Regulates Histone Arginine Methylation Levels via Demethyliminination. *Science.* **306**: 279-283
58. Guimaraes-Costa, A.B., Rochael, N.C., Oliveira, F., Echevarria-Lima, J., and Saraiva, E.M., (2017) Neutrophil Extracellular Traps Reprogram IL-4/GM-CSF-Induced Monocyte Differentiation to Anti-inflammatory Macrophages. *Front Immunol.* **8**: 523
59. Kim, J.G., Lee, J., Roe, J., Tromberg, B.J., Brenner, M., and Walters, T.J., (2009) Hemodynamic changes in rat leg muscles during tourniquet-induced ischemia-reperfusion injury observed by near-infrared spectroscopy. *Physiol Meas.* **30**(7): 529-40

60. Walters, T.J., Kragh, J.F., and Baer, D.G., (2008) Influence of fiber-type composition on recovery from tourniquet-induced skeletal muscle ischemia-reperfusion injury. *Appl Physiol Nutr Metab.* **33**(2): 272-81
61. Willis, R., Seif, A.M., McGwin, G., Jr., Martinez-Martinez, L.A., Gonzalez, E.B., Dang, N., Papalardo, E., Liu, J., Vila, L.M., Reveille, J.D., Alarcon, G.S., and Pierangeli, S.S., (2012) Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort. *Lupus.* **21**(8): 830-5
62. Casado, E., Gratacos, J., Tolosa, C., Martinez, J.M., Ojanguren, I., Ariza, A., Real, J., Sanjuan, A., and Larrosa, M., (2006) Antimalarial myopathy: an underdiagnosed complication? Prospective longitudinal study of 119 patients. *Ann Rheum Dis.* **65**(3): 385-90
63. Taylor, R.W., Schaefer, A.M., Barron, M.J., McFarland, R., and Turnbull, D.M., (2004) The diagnosis of mitochondrial muscle disease. *Neuromuscul Disord.* **14**(4): 237-45
64. Thomas, M.M., Wang, D.C., D'Souza, D.M., Krause, M.P., Layne, A.S., Criswell, D.S., O'Neill, H.M., Connor, M.K., Anderson, J.E., Kemp, B.E., Steinberg, G.R., and Hawke, T.J., (2014) Muscle-specific AMPK beta1beta2-null mice display a myopathy due to loss of capillary density in nonpostural muscles. *FASEB J.* **28**(5): 2098-107
65. Foglia, B., Cannito, S., Bocca, C., Parola, M., and Novo, E., (2019) ERK Pathway in Activated, Myofibroblast-Like, Hepatic Stellate Cells: A Critical Signaling Crossroad Sustaining Liver Fibrosis. *Int J Mol Sci.* **20**(11)
66. Kim, J., Kim, B., Kim, S.M., Yang, C.E., Song, S.Y., Lee, W.J., and Lee, J.H., (2019) Hypoxia-Induced Epithelial-To-Mesenchymal Transition Mediates Fibroblast Abnormalities via ERK Activation in Cutaneous Wound Healing. *Int J Mol Sci.* **20**(10)
67. Muchir, A., Kim, Y.J., Reilly, S.A., Wu, W., Choi, J.C., and Worman, H.J., (2013) Inhibition of extracellular signal-regulated kinase 1/2 signaling has beneficial effects on skeletal muscle in a mouse model of Emery-Dreifuss muscular dystrophy caused by lamin A/C gene mutation. *Skelet Muscle.* **3**(17): 1-10
68. Olson, L.E. and Soriano, P., (2009) Increased PDGFRalpha activation disrupts connective tissue development and drives systemic fibrosis. *Dev Cell.* **16**(2): 303-13
69. Wosczyzna, M.N. and Rando, T.A., (2018) A Muscle Stem Cell Support Group: Coordinated Cellular Responses in Muscle Regeneration. *Dev Cell.* **46**(2): 135-143
70. Feng, J., Zhang, Q., Mo, W., Wu, L., Li, S., Li, J., Liu, T., Xu, S., Fan, X., and Guo, C., (2017) Salidroside pretreatment attenuates apoptosis and autophagy during hepatic ischemia-reperfusion injury by inhibiting the mitogen-activated protein kinase pathway in mice. *Drug Des Devel Ther.* **11**: 1989-2006
71. Thurairajah, K., Briggs, G.D., and Balogh, Z.J., (2018) The source of cell-free mitochondrial DNA in trauma and potential therapeutic strategies. *Eur J Trauma Emerg Surg.* **44**(3): 325-334
72. Braian, C., Hoge, V., and Stendahl, O., (2013) Mycobacterium tuberculosis- induced neutrophil extracellular traps activate human macrophages. *J Innate Immun.* **5**(6): 591-602
73. Godsell, J., Rudloff, I., Kandane-Rathnayake, R., Hoi, A., Nold, M.F., Morand, E.F., and Harris, J., (2016) Clinical associations of IL-10 and IL-37 in systemic lupus erythematosus. *Sci Rep.* **6**: 34604
74. Manzenreiter, R., Kienberger, F., Marcos, V., Schilcher, K., Krautgartner, W.D., Obermayer, A., Huml, M., Stoiber, W., Hector, A., Griese, M., Hannig, M., Studnicka, M., Vitkov, L., and Hartl, D., (2012) Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. *J Cyst Fibros.* **11**(2): 84-92
75. Cepika, A.M., Soldo Juresa, D., Morovic Vergles, J., Malenica, B., Santak, M., Kapitanovic, S., Mayer, M., Anic, B., Sentic, M., and Gagro, A., (2012) Decrease in circulating DNA, IL-10 and BAFF levels in newly-diagnosed SLE patients after corticosteroid and chloroquine treatment. *Cell Immunol.* **276**(1-2): 196-203
76. Jang, C.H., Choi, J.H., Byun, M.S., and Jue, D.M., (2006) Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. *Rheumatology (Oxford).* **45**(6): 703-10

77. Cook, K.L., Warri, A., Soto-Pantoja, D.R., Clarke, P.A., Cruz, M.I., Zwart, A., and Clarke, R., (2014) Hydroxychloroquine inhibits autophagy to potentiate antiestrogen responsiveness in ER+ breast cancer. *Clin Cancer Res.* **20**(12): 3222-32
78. Ramser, B., Kokot, A., Metze, D., Weiss, N., Luger, T.A., and Bohm, M., (2009) Hydroxychloroquine modulates metabolic activity and proliferation and induces autophagic cell death of human dermal fibroblasts. *J Invest Dermatol.* **129**(10): 2419-26
79. Avina-Zubieta, J.A., Johnson, E.S., Suarez-Almazor, M.E., and Russell, A.S., (1995) Incidence of Myopathy in Patients Treated with Antimalarials. A Report of Three Cases and a Review of the Literature. *Br J Rheumatol.* **34**
80. Khosa, S., Khanlou, N., Khosa, G.S., and Mishra, S.K., (2018) Hydroxychloroquine-induced autophagic vacuolar myopathy with mitochondrial abnormalities. *Neuropathology.* **38**(6): 646-652

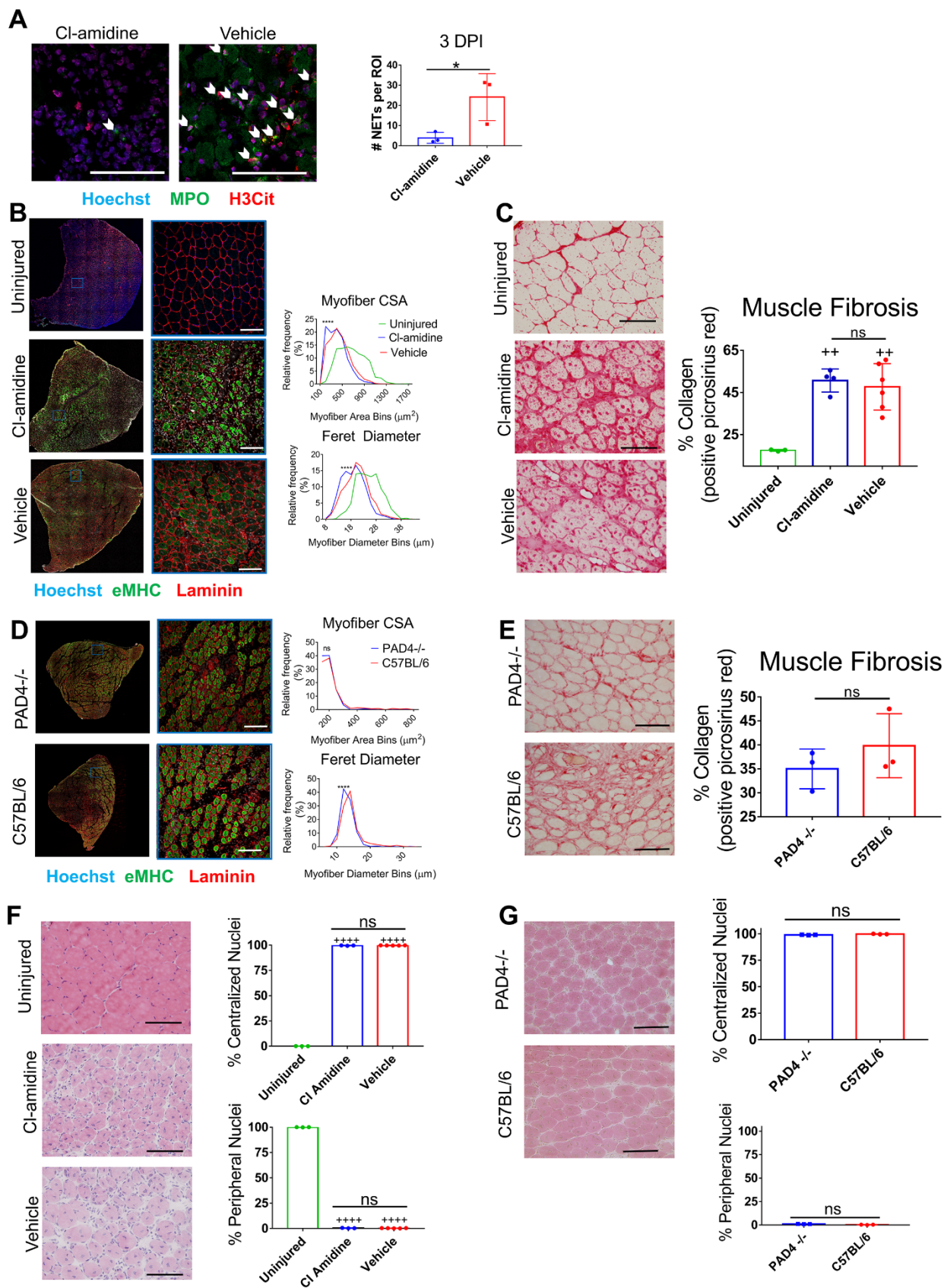


Figure 1. Muscle fibrosis is independent of PAD4 mediated NETosis. *A*) Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with Cl-

amidine or vehicle. Treatment with Cl-amidine significantly decreased NET formation. *B*) Representative images of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle stained with eMHC and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber cross sectional area (CSA) and Feret diameter at 7 DPI, indicating muscles with Cl-amidine treatment have significantly increased frequency of smaller myofibers as indicated by CSA or diameter on x-axis. *C*) Representative histological sections of picrosirius red staining at 7 DPI of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle. ImageJ quantification of positive picrosirius red staining in the same groups. *D*) Representative sections of PAD4^{-/-} mice or C57BL/6 wild type control injured with IR+ctx stained with eMHC and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter at 7 DPI, indicating PAD4^{-/-} muscles have significantly increased frequency of smaller diameter myofibers. *E*) Representative histological sections of picrosirius red staining at 7 DPI of PAD4^{-/-} or C57BL/6 wild type control injured with IR+ctx. ImageJ quantification of positive picrosirius red staining in the same groups. *F*) Representative images of H&E stained TA 7 DPI of uninjured and treated with Cl-amidine or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. *G*) Representative images of H&E stained muscle 7 DPI of PAD4^{-/-} and C57BL/6 mice. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. + indicates difference compared to uninjured group. All scale bars indicate 100 μ m. * indicates $P < 0.05$; ++ indicates $P < 0.005$; **** and +++++ indicate $P < 0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means $\pm$ standard deviation. Each point represents one animal.

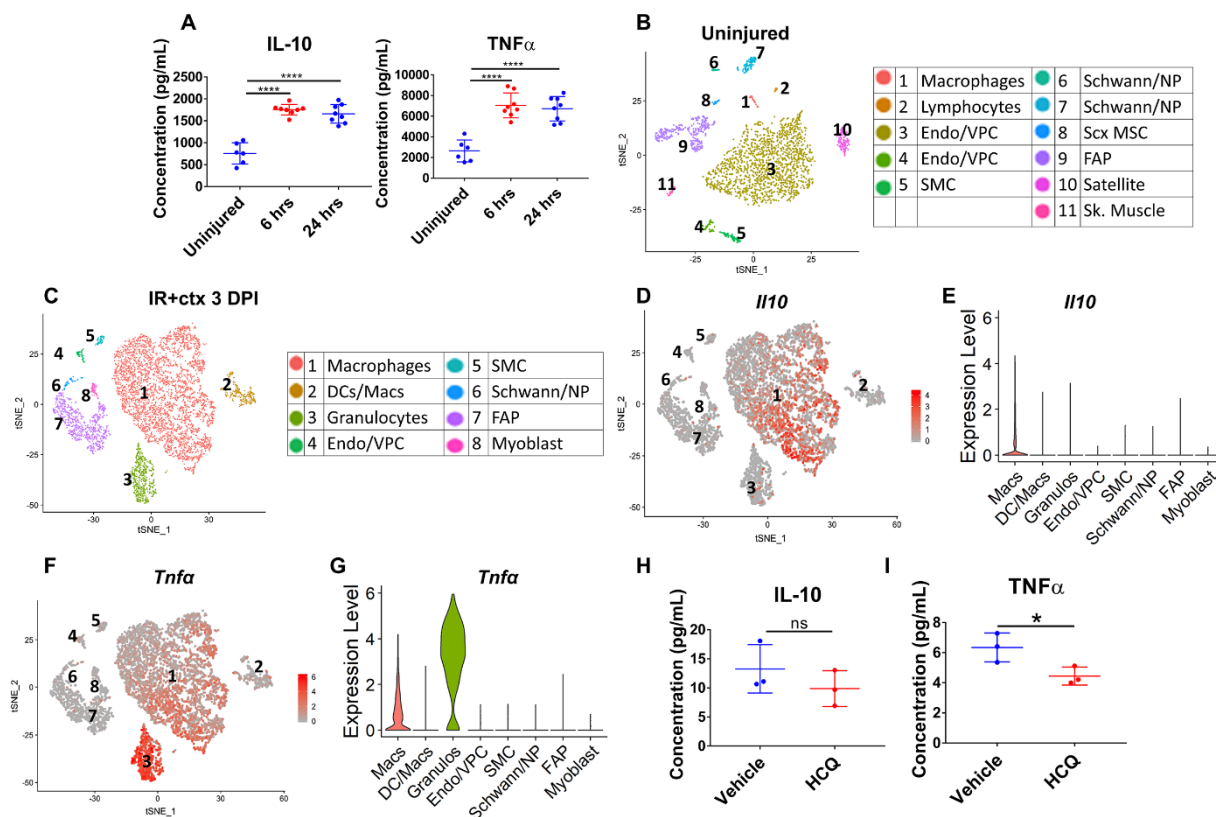


Figure 2. HCQ decreases IL-10 and TNF α in an IR injury model. *A)* In a rat model of tourniquet-induced hind limb IR injury, serum levels of IL-10 and TNF α are significantly elevated at 6 and 24 hr post injury compared to uninjured levels (n=6-8/group, one-way ANOVA with Tukey test). *B)* Clusters resulting from unsupervised clustering in mouse uninjured TA muscle, overlaid letters correspond to adjacent table to indicate cluster identification. *C)* Clusters resulting from unsupervised clustering in mouse IR+ctx injured muscle at 3 DPI, overlaid letters correspond to adjacent table to indicate cluster identification. Violin plots indicate distribution of expression between clusters of cells at 3 DPI of *D-E) Il10* and *F-G) Tnfa*. HCQ lowers circulating *H)* IL-10 and *I)* TNF α levels at 3 DPI in the C57BL/6 mouse model of IR injury (n=3 per group, unpaired *t*-test). * indicates $P<0.05$, **** indicates $P<0.0001$. Each point represents one animal.

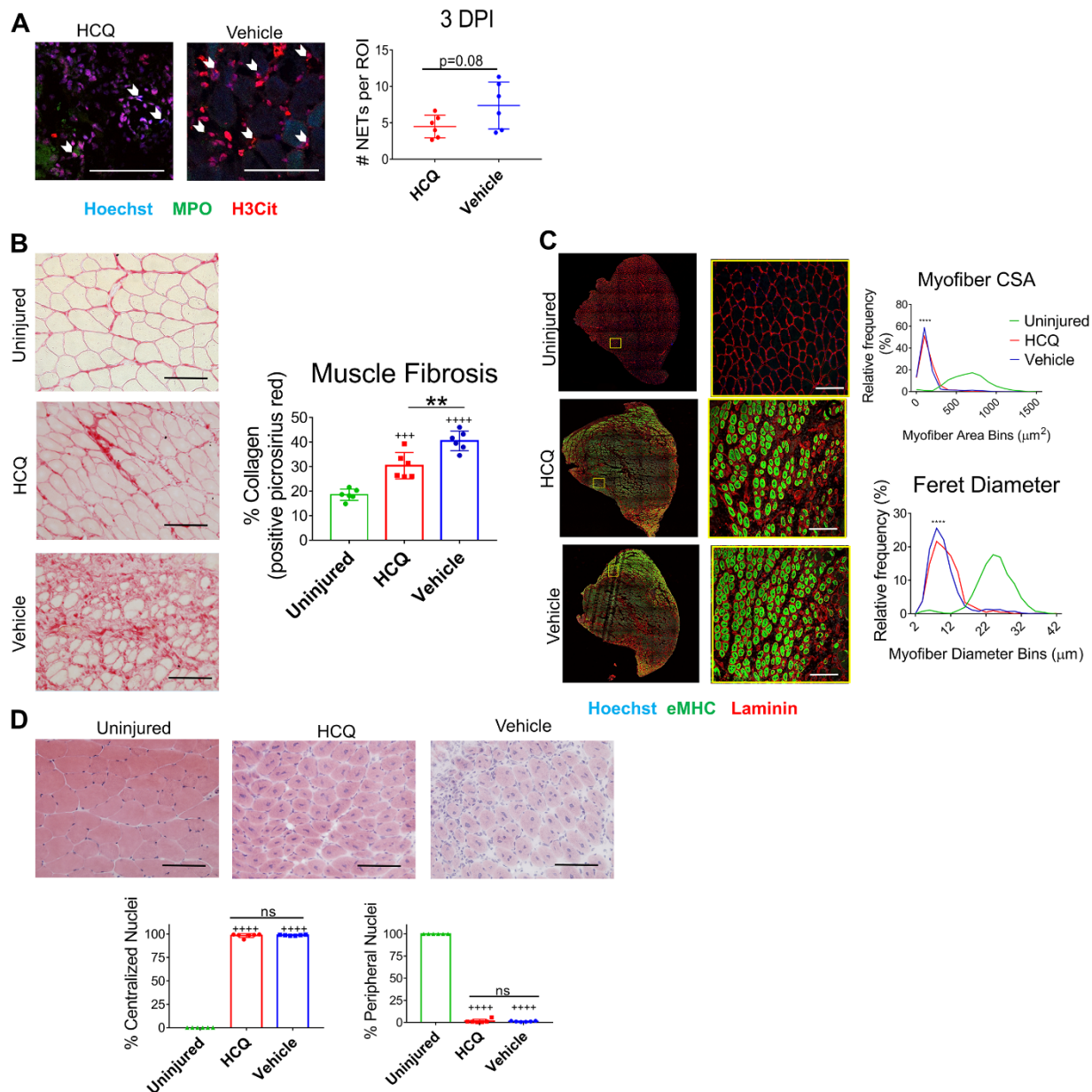


Figure 3. HCQ decreases muscle fibrosis following IR injury. *A)* Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with HCQ or vehicle.

Quantification shows HCQ treatment modestly decreased NET formation. *B)* Representative histological sections of picrosirius red staining of TA at 7 DPI of C57BL/6 mice uninjured and treated with HCQ or vehicle. ImageJ quantification of positive picrosirius red staining in the same groups shows HCQ treatment decreased collagen deposition. *C)* Representative images of C57BL/6 mice uninjured TA and treated with HCQ or vehicle stained with eMHC and laminin at 5 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter, indicating muscles with vehicle treatment have significantly increased frequency of small CSA and feret diameter. *D)* Representative images of H&E stained TA 7 DPI in uninjured and treated with HCQ or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. All scale bars indicate 100 μ m. + indicates difference compared to uninjured group. ** indicates $P<0.01$; ++ indicates $P<0.005$; **** and ++++ indicate $P<0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means $\pm$ standard deviation. Each point represents one animal.

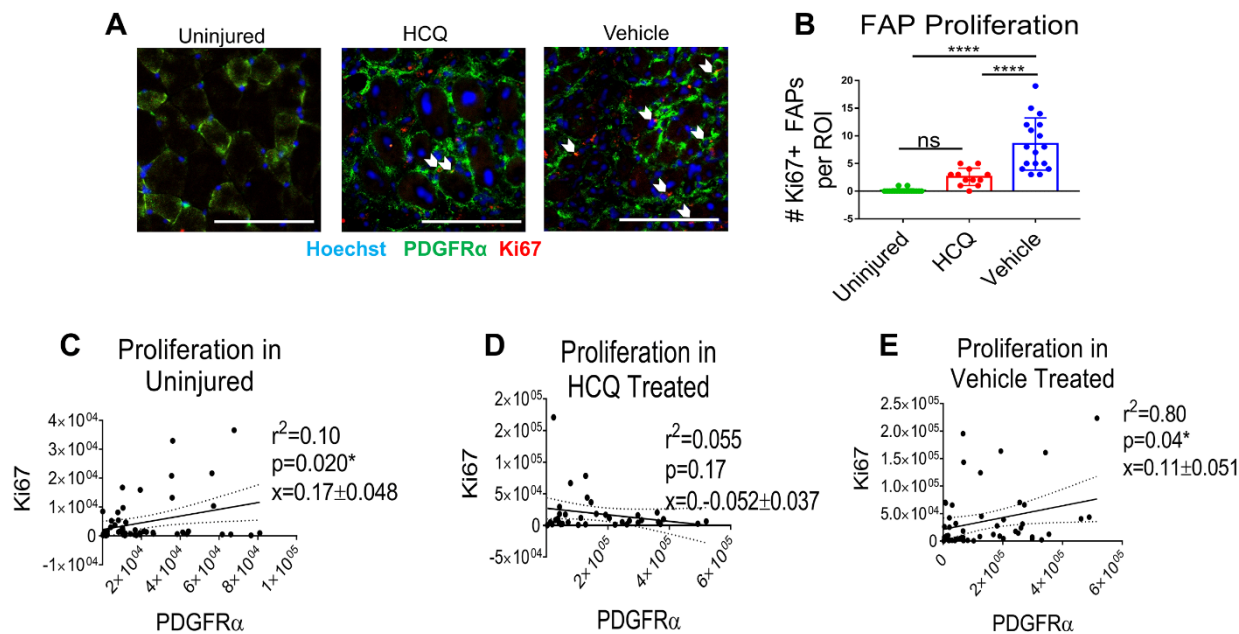


Figure 4. HCQ treatment decreases FAP proliferation. *A)* Immunofluorescent staining of FAPs co-stained with Ki67 (examples indicated by white arrowheads) in the TA at 5 DPI in C57BL/6 mice uninjured and treated with HCQ or vehicle. *B)* Quantification of number of FAPs expressing Ki67. HCQ treatment significantly reduces the number of FAPs expressing Ki67. Correlation curves of total PDGFRα+ and Ki67+ area in *C)* uninjured, *D)* HCQ treated, *E)* vehicle treated TA muscles. All scale bars indicate 100 μ m *indicates $P<0.05$; ****indicates $P<0.0001$. Each point represents one ROI.

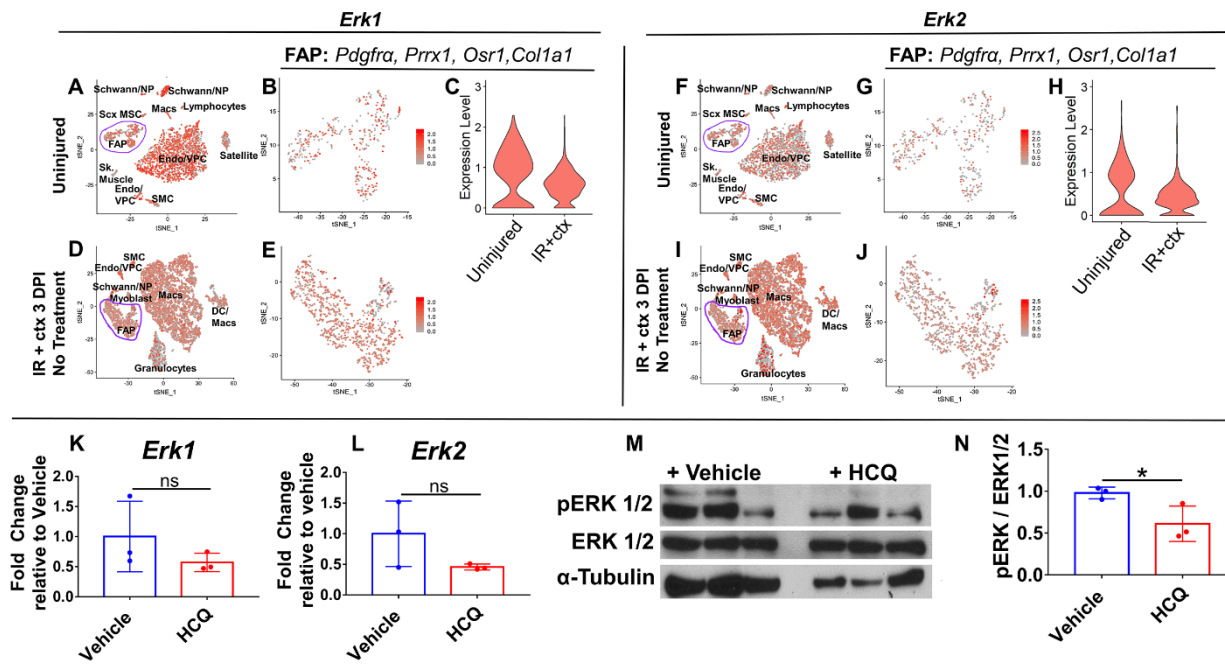


Figure 5. HCQ treatment decreases ERK1/2 phosphorylation in IR injury model. A) Single-cell RNAseq expression of *Erk1* in all cells of uninjured TA. B) Expression of *Erk1* in FAP population of uninjured TA. C) Comparison of *Erk1* expression in FAP population of uninjured and IR+ctx FAP populations. D) Single-cell RNAseq expression of *Erk1* in all cells of IR+ctx injured TA without treatment at 3 DPI. E) Expression of *Erk1* in FAP population of IR+ctx injured TA without treatment at 3 DPI. F) Single-cell RNAseq expression of *Erk2* in all cells of uninjured TA. G) Expression of *Erk2* in FAP population of uninjured TA. H) Comparison of *Erk2* expression in FAP population of uninjured and IR+ctx FAP populations. I) Single-cell RNAseq expression of *Erk2* in all cells of IR+ctx injured TA without treatment at 3 DPI. J) Expression of *Erk2* in FAP population of IR+ctx injured TA without treatment at 3 DPI. Gene expression of K) *Erk1* and L) *Erk2* in the TA at 7 days post IR injury in mice treated with vehicle or HCQ. HCQ treatment does not significantly change *Erk2* or *Erk1* gene expression. Bars indicate fold change in gene expression relative to vehicle treatment. M) Western blot indicating levels of ERK1/2, phosphorylated ERK1/2 (pERK1/2), and alpha tubulin at 7 DPI in TA treated with vehicle or HCQ. N) Corresponding quantification of pERK1/2 relative to ERK1/2 shows significant decrease in ERK1/2 phosphorylation after HCQ treatment. * indicates $P < 0.05$. Bars represent means $\pm$ standard deviation. Each point in K) –N) represents one animal.

Acknowledgements

We would like to acknowledge current and former members of the Levi lab at University of Michigan for thoughtful discussions. We also wish to acknowledge: K. Kessell and members of University of Michigan and Walter Reed Army Institute of Research Unit for Laboratory Animal Medicine for excellent colony

management and animal care and the University of Michigan Microscopy Core for expert advice in microscopy presented here.

Author Contributions

N. Edwards, B. Levi designed research; N. Edwards, C. Hwang, P. Spreadborough, C. Pagani, S. Marini, C. Rowe, P. Yu, A. Mei, N. Visser performed experiments; N. Edwards, S. Marini, C. Hwang analyzed data; A. Huber, A. Strong, G. Hespe, S. Li, J. Knight, M. Shelef, T. Davis, B. Levi provided intellectual contributions and critical review; N. Edwards and B. Levi wrote the paper with input and final approval from all authors.

Disclosures

The authors have no relevant disclosures.

Disclaimers

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Funding Sources

N.J.E. was supported by a Ruth L. Kirschstein Institutional National Research Service Award Postdoctoral fellowship (T32-HD007505). C.H. was supported by a Howard Hughes Medical Institute Medical Research Fellowship. M.S. was supported by National Institutes of Health NIAMS K08 AR065500. B.L. was supported by National Institutes of Health (NIH)/National Institute of General Medical Sciences Grant K08GM109105, NIH Grant R01GM123069, an American College of Surgeons Clowes Award, U.S. Department of Defense Grants W81XWH-18-1-0653 (OR170174) and W81XWH-17-1-0655 (OR160105).